Universität Rostock Traditio et Innovatio

Novel small molecules as GSK-3β inhibitors in human neural progenitor cells

Dissertation

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von

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geb. am 03.02.1983 in Köln

Rostock, 02.12.2010

urn:nbn:de:gbv:28-diss2011-0014-9

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Tag des mündlichen Kolloquiums: 21.03.2011

Erklärung

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Rostock, 26.11.2010

Content

Abbreviations

%	percent
°C	degree Celsius
µ	micro
ATP	adenosine triphosphate
BCL	B-cell lymphoma 9
bFGF	basic fibroblast growth factors
BIO	6-Bromoindirubin-3'-oxine
BSA	bovine serum albumine
CBP	CREB binding protein
CCND1	cyclin D1
cDNA	copy DNA
CK	casein kinase
CMYC	c-myc
CNS	central nervous system
CRMP	collapsing response mediator protein 2
C_t	cycle threshold
D	day(s)
DAAM	Dishevelled-associated activator of morphogenesis 1
DAPI	4', 6-Diamidine-2-Phenylindole
DISC1	disrupted in schizophrenia 1
Dkk1	Dickkopf1
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
Dvl	dishevelled
E	embryonic stage
EDTA	ethylendiamin-tetraacetat
EGTA	ethylene glycol tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunoabsorbent assay
ES cells	embryonic stem cells
Et al.	et alii
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
Fig.	figure
FSC	forward scatter
Fz	frizzled
g	gram, acceleration of gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase
h	hour(s), human
HBSS	Hanks' balanced salt solution
I3M	indirubine-3'-monoxime
IC	immunocytochemistry

iPSC	induced pluripotent stem cells
JNK	c-Jun N-terminal kinase
k	kilo
KP	Kenpaullone
l	liter
LRP	LDL receptor related proteins
LEF	lymphoid enhancer factor
m	milli, mouse
MAP2	microtubule-associated protein 2
MEK	MAP/ERK pathway
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
n	nano
Na	sodium
NFAT	nuclear factor of activated T-cells
NPC	neural progenitor cell
OD	optical density
PBS	phosphate buffered saline
pc	post coitum
PFA	paraformaldehyde
PCP	planar cell polarity
PCR	polymerase chain reaction
PI	propidium iodide
PKC	protein kinase C
Pygo	Pygopus
Rac	Ras-related C3 botulinum toxine substrate
RhoA	Ras homologous A
RNA	ribonucleic acid
RNase	ribonuclease
ROCK	Rho-associated kinase
Ror	orphan tyrosine kinase
rpm	rotations per minute
RT	reverse transcriptase
s	seconds
SB216763	3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione
SDS	sodium dodecylsulphate
SEM	standard error of mean
SHH	sonic hedgehog
SSC	sideward scatter
SVZ	subventricular zone
Tab.	table
TCF	T-cell enhanced factor
TSC-2	tuberous sclerosis complex 2
TrCP	Tranducin repeat-containing protein
Tris	Tris-(hydroxymethyl-)aminomethane

TUBB3	βIII-tubulin
WIF	Wnt inhibitory factor

1 Introduction

1.1 Stem cells

Stem cells are defined as cells with the ability for self-renewal and differentiation into other cell types (Weissmann et al., 2000; Cai et al., 2004). This can be achieved by symmetric cell division (proliferation) and asymmetric cell division (differentiation) (Martin-Rendon and Watt, 2003). Regarding the potency of the cells to develop into other cell types they are classified as totipotent, multipotent and pluripotent. Totipotent cells can differentiate into all cell types of an organism and originate from a fertilized oocyte up to its forth cell division. A pluripotent or embryonic stem cell is derived from the inner mass of a blastocyst and can differentiate into cells of all three germ layers: endoderm, ectoderm and mesoderm (Loebel et al., 2003). Multipotent, adult stem cells or progenitor cells can be found in different adult tissues. Their differentiation is limited to the cells of the tissue they originate from. (Körbling and Estrov, 2003).

1.1.1 Neural progenitor cells and neurogenesis

During embryogenesis, the neural plate is developed out of the ectoderm of the blastocyst, which later on forms the neural tube. Forebrain and midbrain arise from the front part of the neural tube, whereas the back part creates the hindbrain and the spinal cord. The differentiation of neural progenitor cells (NPC) into neurons or glia cells is initiated by signals from the mesoderm (Kandel et al., 2000).

Neural progenitor cells or neural stem cells can be found in different brain regions, such as the hypothalamus, the dentate gyrus of the hippocampus and the forebrain, the structure of the subventricular zones of the lateral ventricle of the olfactory bulb and the subgranular zone of the dentate gyrus (Temple and Alvarez-Byulla, 1999). NPC can be used for proliferation and differentiation studies and can be obtained either from embryonic stem cells (Zhang et al., 2001; Schuldiner et al., 2001; Kim et al., 2007) or

directly isolated from fetal or adult tissue (Ling et al., 1998; Akiyama et al, 2001; Arsenijevic et al., 2001).

Whether these cells give rise to neurons or glia cells is dependent on the stage of development, since the asymmetric cell division around E13 leads in mice to an increase of neurons, whereas asymmetric cell divisions at stage 18 pc (post coitum) and early postnatal stages form more glial cells (Hirabayashi and Gotoh, 2005).

To cultivate NPCs *in vitro*, either mitogens or immortalization via the transduction of an immortalized oncogene are used. This promotes standardized culturing conditions. Primary cell lines are limited regarding their availability (Palmer et al., 2001) whereas immortalized cells might display different behavior due to the presence of the oncogene, but they obtain a stable genotype and phenotype (Dang et al., 1999; Kim 2004).

Several immortalized cell lines have been published: V-myc propagated NPC lines include C17.2, derived from mouse cerebellum (Ryder et al., 1990), H6, derived from human 15-week-old telencephalon (Flax et al., 1998), HNSC.100, derived from human 10-week-old forebrain (Villa et al., 2002), Mes2, derived from human 8-week-old VM (Lotharius et al., 2002). In this study, a human neural progenitor cell line ReNcell VM, derived from the ventral midbrain of a 10-week-old fetus, has been used. Detailed information about this cell line will be given in paragraph 1.4.

To obtain a proliferating state, the addition of growth factors to the cultivation medium is required, the most common ones are the epidermal growth factor (EGF) or the fibroblast growth factor (FGF)-2 (Tropepe et al., 1999). The withdrawal of growth factors induces differentiation into a region-appropriate progeny (Potter et al., 1999).

1.2 Wnt signaling

Wnt signaling pathways are involved in various cellular processes such as proliferation, cell death, cell polarity, morphogenic movements or self-renewal (Moon et al., 2004). Up to now, three different intracellular pathways have been known where a Wnt ligand binds to a Fzd receptor.

1. Wnt/β-catenin pathway (“canonical” pathway)
2. PCP (planar cell polarity) pathway
3. Wnt/Ca^{2+} pathway

1.2.1 Wnt/β-catenin pathway

The Wnt/β-catenin pathway is one of the best described pathways. It is mainly characterized by β-catenin and its degradation (inactive state) or accumulation (active state). β-catenin is a constitutively expressed protein which is involved in cell adhesion. It connects cadherin cell adhesion molecules to the cytoskeleton (Aberle et al., 1996).

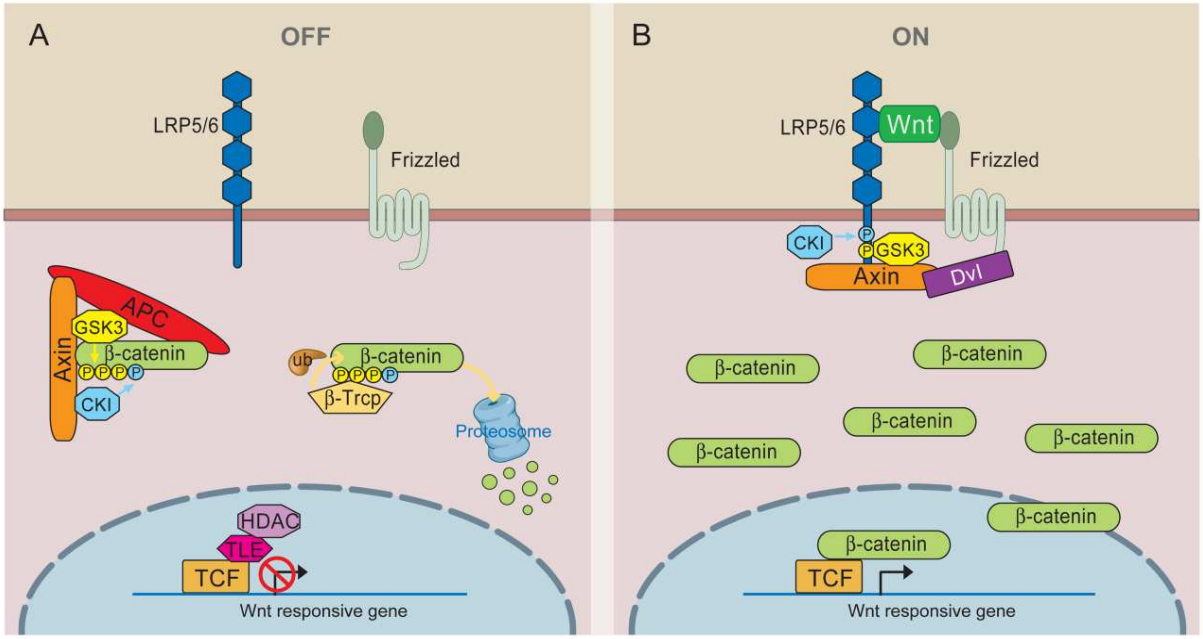

Figure 1: Overview of Wnt/β-catenin signaling
A) In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3 and CK1, and is phosphorylated by CK1 (blue) and subsequently by GSK3 (yellow). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenin for proteosomal degradation. Wnt target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDAC). B) In the presence of a Wnt ligand, a receptor complex forms between Fzd and LRP5/6. Dvl recruitment by Fzd leads to LRP5/6 phosphorylation and Axin recruitment. This disrupts Axin-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it serves as a co-activator for TCF to activate Wnt responsive genes. (MacDonald et al., 2009)

Inactive state

In an inactive state, β-catenin is phosphorylated by a degradation-complex and subsequently degraded in the cytosol. The degradation complex consists of GSK-3β, APC

and, Axin and CK1. GSK-3β and CK1 phosphorylate β-catenin at its amino-terminal region, at which CK1α is responsible for phosphorylation at Ser45 and subsequently GSK-3β for phosphorylation at threonine 41, serine 37 and serine 33 (Kimelman and Xu, 2006). The phosphorylation of Serine 37 and 33 forms a binding site for β-TrCP (transducin repeat-containing protein), which is an E3 ubiquitin ligase unit. This enzyme ubiquitinates β-catenin, which is then degraded by proteosomes in the cytosol (Fig. 1a) (He et al., 2004). Beside GSK-3β, Axin and APC are also crucial for the degradation process. Both proteins are also phosphorylated by GSK-3β and CK1, which causes an increased interaction of Axin and APC with β-catenin, subsequently resulting in an enhanced phosphorylation/degradation of β-catenin (Kimelman and Xu, 2006; Huang and He, 2008).

Active State

For activation, two receptor families are required: frizzled seven-pass transmembrane receptors (Fzd) (Logan and Nusse 2004) and LDL receptor related proteins 5 and 6 (LRP5 and 6) (He et al, 2004). The family of 10 Fzd receptors are composed of seven transmembrane domains and an extracellular conserved cystein rich domaine, which is crucial for Wnt ligand binding. LRP6 is not only essential for activation of Wnt/β-Catenin signaling, but can also inhibit the non-canonical pathways *in vivo*, whereas the mechanism has not been well described yet. Bryja et al. (2009) described a competing mechanism for Wnt ligands, while Tahinci and co-workers (2007) elucidated the effect but not the mechanism. Fzd receptors play a role in both signaling pathways, canonical and non-canonical ones (Medina et al., 2000).

In addition to these two main receptor families, other receptors have been described. Ryk, an atypical tyrosine kinase (Hsieh et al., 1999) and Ror2 are not necessary for Wnt/β-catenin signaling, but they can inhibit the pathway (van Amerogen et al., 2008) by binding the Wnt inhibitory factor (WIF) or activating c-Jun N-terminal kinase (JNK), respectively.

Upon binding of a Wnt ligand to the receptors, LRP6 is phosphorylated by GSK-3β and CK1α (Tamai et al, 2004). Cytoplasmic Dishevelled (Dvl) is recruited to the membrane and interacts with Fzd, which is not well understood yet but could be triggered by the activation of heterotrimeric G-proteins (Cadigan and Liu, 2006). Axin now binds to the dual phosphorylated PPPSPxS motifs of LRP6 (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2005), resulting in the disruption of the degradation complex (Fig. 1b). In

addition, the phosphorylated LRP6 cytoplasmic domain or individual phospho-PPPSPxS peptides can directly inhibit GSK-3β phosphorylation of β-catenin *in vitro* (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009).

β-catenin is now not longer degraded but translocated in the nucleus. This mechanism is poorly understood (Henderson and Fagotto, 2002; Stadeli et al., 2006). β-catenin possesses no nuclear localization signal, which would trigger the nuclear localization (Henderson and Fagotto, 2002). Wu et al. (2008) described that the β-catenin accumulation induced by Wnt-ligand binding is not effectual enough for the translocation process. They suggested that Wnt activation of Rac1 GTPase is required as well.
In the nucleus, β-Catenin binds to the TCF (T-cell factor) /LEF (lymphoid enhancer binding factor) family of DNA-bound transcription factors (Fig. 1b) (Arce et al., 2006; Hoppler and Kavanagh, 2007). It replaces the repressor Groucho, promotes histone deacetylation and chromatin compaction (Daniels and Weis, 2005) and recruits co-activators such as CBP (CREB binding protein), BCL (B-cell lymphoma 9) and Pygo (Pygopus) (Klaus und Birchmeier, 2008). By this process, the transcription of Wnt-target genes is activated, whereas Wnt target genes are divers (Vlad et al., 2008) and cell- and context dependent (Logan and Nusse, 2004). For an updated list, see the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html. It is noteworthy that many genes of Wnt signaling components are regulated by TCF/β-catenin (Chamorro et al., 2005; Kazanskaya et al., 2004; Khan et al., 2007; Logan and Nusse, 2004).

1.2.2 Non-canonical Wnt pathways

Non-canonical pathways require the binding of a Wnt ligand to a Fzd receptor but are independent of β-catenin. They are involved in planar cell polarity (PCP), gastrulation, migration, convergent extension and transcription (Schambony and Wedlich, 2007). Two β-catenin independent pathways are described, namely the Wnt/PCP and the Wnt/Ca^{2+} pathway.

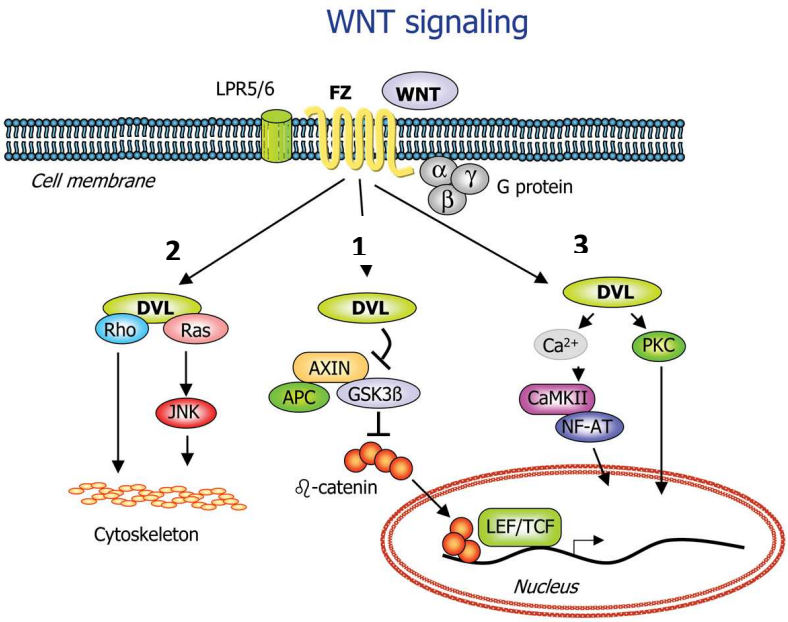

Figure 2: Wnt Pathways. The extracellular signaling molecule WNT activates three pathways: (1) Early cell fate decisions are controlled via the *canonical pathway* (middle): it comprises the regulation of gene expression by inducing β-catenin-mediated transcriptional activation. Interaction of WNT with the transmembrane receptor frizzled (FZD) activates dishevelled (DVL), which induces the disassembly of a complex consisting of Axin, adenomatosis polysis coli (APC), glycogen synthase kinase 3β (GSK3β) and β-catenin. In non-stimulated cells, GSK3β phosphorylates β-catenin, thereby triggering its degradation. Activitation of the pathway effectively increases the levels of β-catenin in the cyctoplasm, which is then translocated to the nucleus. Here it forms the β-catenin-T-cell specific transcription factor complex that activates the transcription of target genes. (2) In the planar cell polarity pathway (left), FZD functions through G-proteins to activate DVL, which thereupon signals to Rho GTPases (Rho or Rac or both). Activated Ras signals through the c-Jun amino (N)-terminal kinase (JNK). Activation of Rho-GTPases induces changes in the cytoskeleton. In neurons, this pathway is involved in dendritic arborization. (3) In the WNT/calcium pathway (right), activation of DVL activates protein kinase C (PKC) and induces the release of intracellular calcium, which activates a calcium/calmodulin-dependent protein kinase II (CaMKII). Klipp and Liebermeister *BMC Neuroscience* 2006 **7**

Wnt/PCP pathway

The Wnt/PCP pathway is responsible for polarization of epithelial cells, e.g. during gastrulation or hair orientation (Zeeman et al., 2003). The binding of a Wnt ligand to Fzd leads, via Dvl, to the activation of JNK and of small GTPases (heterotrimeric G proteins) Rac (Ras-related C3 botulinum toxine substrate) and RhoA (Ras homologous A) (Wallingford and Habas, 2005). Rac activation is followed by a signaling cascade which subsequently activates DAAM1 and RhoGTPase (Habas et al., 2001) which in turn activates ROCK (Rho-associated kinase) (Fig. 2.2). This part of Wnt/PCP signaling is associated to focal adhesions and the rearrangement of the actin cytoskeleton (Ridley and Hall, 1992; Leung et al., 1995). The Rac branch can activate JNK independently of

DAAM1 (Disheveled-associated activator of morphogenesis 1) which influences cytoskeleton dynamics (Wallingford and Habas, 2005). Known ligands for activation of this pathway are Wnt 4, 5a and 11.

Wnt/Ca^{2+} pathway

The overexpression of Wnt 5a or 11 has been shown to increase the intracellular Ca-concentration without influencing the expression of β-catenin (Slusarski et al., 1997b), first indicating that there might be a signaling pathway that includes the binding of a Wnt ligand to a Fzd receptor, but without any involvement of β-catenin. When a Wnt ligand binds to Fzd and heterodimeric G-proteins, diacylglyerol and IP3 are generated and later on calcium fluxes (Sheldahl et al., 2003). The key enzyme in the pathway is CamKII (calcium/calmodulin-dependent kinase), which is activated by calcium ions as well as PKC (protein kinase C) (Kuhl et al., 2000). Active CamKII can control the transcription Factor NFAT (nuclear factor of activated T-cells), which is, similar to β-catenin, translocated to the nucleus where it regulates the transcription of target genes (Fig. 2.3) (Dejmek et al., 2006).

1.2.3 GSK-3

GSK-3 is, together with β-catenin, the key player of the Wnt/β-catenin pathway. It was originally identified as a protein kinase which is involved in the glycogen metabolism, which was soon followed by the investigation of its importance for Wnt signaling. GSK-3 is encoded in mammals by two genes, GSK-3α and GSK-β. They share an almost complete sequence identity of their protein kinase domains, but differ in their *N*- and *C*-terminal regions (Woodgett, 1990). Two different splicing variants of GSK-3β are described in humans and rodents, GSK-3β1 and GSK-3β2. GSK-3β1 is expressed ubiquitously, whereas GSK-3β2 is expressed specifically in the nervous system, especially during development (Mukai et al., 2002). Recently, a role for this splicing variant during neuronal morphogenesis has been described (Wood-Kaczmar et al., 2009; Castano et al., 2010). In addition to β-catenin a plethora of substrates for GSK-3β are described, including proteins which are involved in metabolism and signaling, structural proteins or transcription factors. GSK-3 is regulated by an inactivation-phosphorylation, GSK-3α at Ser21 (Serin21),

GSK-3β at Ser9. The activation phosphorylation is within the T-Loop at Y279/216 (GSK-3α and β, respectively) (Hughes et al., 1993).

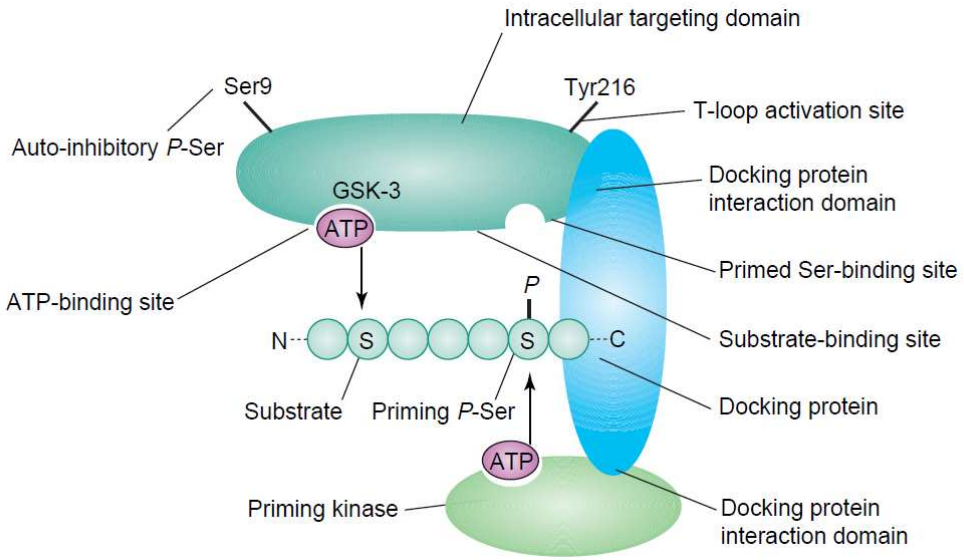

Figure 3: Potential sites for inhibition of glycogen synthase kinase 3 (GSK-3). Most kinase inhibitors act by competition with either ATP or metal-binding sites that are directly involved in the catalytic process. However, small-molecular-weight compounds might regulate GSK-3 activity by inhibiting the protein–protein interactions that are necessary for the binding of substrate [the primed phosphorylated serine binding area and the docking protein (Axin and presenilin)], by modulating the Tyr216 (GSK-3b) and Tyr279 (GSK-3a) activation sites and the Ser9 (GSK-3b) and Ser21 (GSK-3a) inhibition sites, and by interfering with the intracellular targeting domain of GSK-3. Inhibition of the interaction between the docking protein and the priming kinase might change the substrate specificity of GSK-3. Meijer et al, 2004

GSK-3 has an important neuronal function as one of its substrate CRMP2 (collapsin response mediator protein2) (Cole et al., 2004; Jiang et al., 2005, Yoshimura et al., 2005). CRMP2 binds to microtubulins. This can be suppressed by GSK-3 via phosphorylation of CRMP2 resulting in increased microtubule polymerization and axonal outgrowth. Another neuronal target is Tau. Tau is a phosphoprotein that is associated with microtubules in normal mature neurons. It supports tubulin-assembling to microtubulins and promotes stabilization of microtubulin structures (Weingarten et al., 1975). Enhanced phosphorylation of Tau leads to neurofibrillary tangle precipitates, which is observed in Alzheimer's disease (AD) (Maccioni et al., 2001). GSK-3 seems to play an important role in AD, as inhibition of GSK-3α results in decreased production of Aβ peptide from amyloid precursor protein (Phiel et al., 2003). GSK-3 is also of interest in schizophrenia, as the characteristic protein DISC1 (disrupted-in-schizophrenia 1) is regulated by GSK-3β/β-catenin signaling (Mao et al., 2009). This protein interacts with GSK-3β and blocks its

activity. Mice lacking DISC1 in the dentate gyrus display schizophrenia- and depression-like symptoms. This behavior was normalized by treatment with a GSK-3 inhibitor (Mao et al., 2009). Other diseases which are linked to GSK-3 are diabetes (Cohen and Goedert, 2004), cancer or bipolar disorders (Gould, 2006). This makes it an attractive pharmacological target. A classic example for a GSK-3β inhibitor as a drug is lithium which has been used as a mood stabilizer for over 50 years (Klein and Melton, 1996). Prominent drugs are SB-216763 (Coghlan et al., 2000), Kenpaullone (Bain et al., 2003; Leost et al., 2000) or 6-Bromoindirubin-3'-oxime (BIO) (Meijer et al., 2003; Polychronopoulos et al., 2004). Most of the described GSK-3 inhibitors act via ATP-competition, whereas Lithium or Beryllium are Mg-competitive (Forde and Dale, 2007). For a detailed review regarding GSK-3, its regulation, substrates and connection to neural development see Forde and Dale, 2007 or Hur and Zhou, 2010.

1.2.4 Wnt signaling and GSK-3 in NPC

The role of Wnt signaling in hNPCs is not well described yet. β-catenin was first identified in the brain in 1998 (Zhang et al., 1998). From then on, a plethora of studies have described the roles of Wnt signaling in NPC. In general, one can say that Wnt participates in nearly all processes which are involved in generating a functional neuron from a neural stem cell, such as neural induction (Wilson et al., 2001), anterior-posterior patterning (Popperl et al., 1997), migration (Chenn and Walsh, 2003), axon guidance (Lu et al., 2004), synaptogenesis (Hall et al., 2000) and dendritogenesis (Yu et al., 2003).

Wnts act on the developing murine CNS. The active form of β-catenin increases the proliferation of the neural progenitor pool, which enlarges the entire neural tube (Chenn and Walsh, 2003). In line with these data, it has been reported that the knock-out of β-catenin decreases the size of different brain regions (Zechner et al., 2003).

In isolated NPC, the stage of origin is of great importance. Murine NPCs from early stages (E8.5-10.5) show an increased proliferation following the canonical Wnt stimulus (Viti et al., 2003), whereas NPCs from later stages (E11.5-13.5) respond with an enhanced neuronal differentiation (Hirabayashi et al., 2004; Muroyama et al., 2004).

The influence of canonical Wnt signaling on dopaminergic differentiation is also of great interest. Different *in vitro* studies describe the influence of different Wnt ligands on

dopaminergic differentiation studies in the midbrain of mice (Castelo-Branco et al., 2003; Schulte et al., 2005). Wnt5a is crucial for the morphogenesis of the ventral midbrain and dopaminergic differentiation of NPC (Andersson et al., 2008). Recent studies describe that β-catenin regulates midbrain dopaminergic differentiation *in vivo* (Joksimovic et al., 2009; Tang et al., 2010).

Several pathways beside Wnt signaling are reported to regulate proliferation or differentiation of neural progenitor cells: Notch (Yoon and Gaiano, 2005), SHH (Sonic hedgehog) (Machold et al., 2003) and FGF (fibroblast growth factor) (Iwata and Hevner, 2009). But interestingly, GSK-3β is involved in all four pathways.

In general, it seems that active GSK-3β promotes neuronal differentiation whereas the deletion of GSK-3 (Kim et al., 2009) or the overexpression of its negative regulators (Mao et al., 2009; Bultje et al., 2009) enhance proliferation of NPCs but inhibit differentiation. Kim and co-workers were able to demonstrate the crucial role for GSK-3 in the development of the nervous system. A double knock-out of both, GSK-3α and β, in mice resulted in an increased cortical surface area with a convoluted shape, which is caused by an over-expansion of NPCs. The reduction of neurons leads to a thinner cortex, compared to control mice. In addition, they reported the phosphorylation status of GSK-3 targets. β-catenin and c-Myc are increased during neuronal differentiation. As these two targets are described as proliferation-promoting factors, these results indicate that active GSK-3 promotes neuronal differentiation.

1.3 Small molecules and stem cells

The research on small molecules has gained more and more attention within the last years. On the one hand, they can optimize culture conditions of cell cultures, on the other hand, they can manipulate cellular processes by inhibiting or activating signaling pathways. Thus, mechanisms of diseases can be studied, drugs can be designed.

Of great interest is the modulation of stem cell fate by influencing the maintenance and differentiation of stem cells. Several small molecules have already been investigated regarding their influence on stem cell differentiation (Borowiak et al., 2009; Zhu et al., 2009; Kim et al., 2009; Schneider et al., 2008).

The inhibition of GSK-3 by small molecules and its influence on stem cell proliferation and differentiation is discussed controversially. It seems that the impact of the Wnt/β-catenin pathway on differentiation is stage and context-specific. Inhibition of GSK-3 by BIO activates the transcription of Oct3/4, Rex-1 and Nanog, which are characteristic for the maintenance of a pluripotent, undifferentiated state in human and mouse ESCs (Sato et al., 2004). CHIR99021 promotes ESCs self renewal in combination with two MEK (MAP/ERK pathway) inhibitors (Ying et al., 2008). In contrast, treatment of NPC with different GSK-3 inhibitors I3M (indirubine-3-monoxime) and KP (kenpaullone) promotes dopaminergic differentiation of rat mecencephalon precursors (Castelo-Branco et al., 2004).

But not only in stem cells small molecules are of interest. Reprogramming of somatic cells to pluripotent cells with the characteristics similar to embryonic stem cells create a new way to study differentiation processes, but also enables research on various diseases. There are several advantages of iPSC (induced pluripotent stem cells) over embryonic stem cells (ESC). The most prominent is the “never-ending source”, but iPSC can also be generated out of patient’s cells, which would be of great benefit regarding transplantation studies. The low reprogramming rates and the need of the four transcription factors which have oncogenic potential are still a big problem (Palma et al., 2008; Wei et al., 2006; Chen et al., 2006; Chiou et al., 2008; Knoepfler, 2008). Small molecules have already been shown to improve the reprogramming efficiency (Shi et al., 2008; Huangfu et al., 2008 a and b; Li et al., 2009) as well as the substitution of up to two transcription factors (Shi et al., 2008; Huangfu et al., 2008 a and b).

Small molecules offer a great potency, varying from standardization of culture conditions to improvement of experimental results.

1.4 Aim of this study

Wnt signaling has been shown to be involved in cellular processes in NPCs such as proliferation and differentiation. In this work, we used a human neural progenitor cell line, ReNcell VM (Millipore, Billerica, USA), which is derived from the ventral midbrain of a 10-week-old fetus and immortalized by v-Myc retroviral transduction. This cell line is

characterized by rapid proliferation and can be easily differentiated into astrocytes, neurons and oligodendrocytes (Donato et al., 2007; Morgan et al., 2009). Previous studies done by our group have shown that this cell line responds to the stimulation of Wnt/β-catenin signaling with increased neuronal differentiation (Hübner et al., 2010).

Potent GSK-3β inhibitors offer a great promise as to the manipulation of Wnt-connected cell proliferation and differentiation. In addition, they could be useful for investigations into, or therapy of any disease that is linked to a malfunction of GSK-3β, such as diabetes, bipolar disorders, or Alzheimer's disease.

Therefore, the aims of this study were

- To establish a multi-step screening system for novel GSK-3β inhibitors
    - How does the Wnt/β-catenin pathway react to GSK-3 inhibition?
    - What are the optimal read-outs for a multi-level screening system?

- To study the influence of GSK-3β inhibition on hNPC
    - What is the influence on hNPC proliferation and differentiation?

Potential novel GSK-3β inhibitors were provided by the Leibniz-Institute for Catalysis (LIKAT), Prof. Dr. Matthias Beller.

2 Material and Methods

2.1 Material

2.1.1 Technical equipment

	System	Company
camera	DS2M	Nikon
cell culture microscope	Eclipse TS100	Nikon
fluorescence microscope	Biozero	Keyence
cell counter	CASY	Roche
incubator		Binder
sterile working bench	Antares 48	Sterile
centrifuge	Z383K	Hermle
centrifuge	Z233MK-2	Hermle
centrifuge	Universal 30 RF	Hettich
balance	MCBA 100	Sartorius
FACS	FACSCalibur	Becton Dickenson
heating block	Thermomixer	eppendorf
luminometer	LB 9508	Berthold
nucleofector	NucleofectorII	Amaxa
pH-meter	Mettler	Toledo
pipets	reference	eppendorf
plate reader	Magellan	Tecan
real-time PCR Cycler	LightCycler 1.5	Roche
vortexer	MS1	IKA

Table 1: Technical equipment.

2.1.2 Chemicals

Routinely used chemicals were purchased with “pro analysis“ grade and were supplied, if not otherwise stated, by Calbiochem, Fluka, Merck, Sigma and Roth.

2.1.3 Buffers

RIPA-buffer (lysis buffer for cell extracts)

20mM Tris pH 7.4
137mM NaCl
0.1% SDS
0.1% sodiumdesoxycholate
1% Triton X-100
10% glycerol
2mM EDTA
1mM EGTA
1mM NaF
20mM sodiumpyrophosphate
plus protease and phosphatase inhibitor cocktail (Roche)

PBS

137nM NaCl
2.7nM KCl
8.1nM Na_2HPO_4
1.5nM KH_2PO_4

Washing buffer (ELISA)

0.05% Tween20 in PBS, pH 7.2-7.4

IC-Diluent #1/Blocking solution (ELISA)

1% BSA in PBS, pH 7.2-7.4

IC-Diluent #4 (ELISA)

1mM EDTA

0.5% Triton X-100 in PBS, pH 7.2-7.4

FACS fixing solution

1% PFA in PBS

FACS saponin buffer

0.5% BSA

0.5% saponin

0.02% NaN_3 in PBS

FACS wash buffer

0.5% BSA

0.02% NaN_3 in PBS

Blocking buffer (immunocytochemistry)

5% normal goat serum

0.3% Triton-X100 in PBS

Antibody incubation buffer (immunocytochemistry)

1% normal goat serum in PBS

2.1.4 Cell culture media, buffers and supplements

DMEM (Dulbecco's Modified Eagle Medium) 4.5g/l glucose	Invitrogen
DMEM/F12	Invitrogen
HBSS (Hank's balanced salt solution)	Invitrogen
FCS (fetal calf serum)	Invitrogen
Pen/Strep 100x	PAA

Gentamycin	Invitrogen
B27	Invitrogen
bFGF	Roche
EGF	Roche
heparin sodium salt	Invitrogen
Trypsin/EDTA	Invitrogen
Trypsin-Inhibitor	Sigma
Trypsin/Benzonase solution	25U/ml Benzonase in Trypsin-EDTA
Trypsin-inhibitor/Benzonase	1% HSA, 25 /ml Benzonase, 0.55mg/ml trypsin-inhibitor in DMEM/F12
Benzonase	Merck
mouse laminin	Trevigen

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cultivation of ReNcell VM

ReNcell VM were initially provided by ReNeuron (Guildford, UK) and can meanwhile be purchased from Millipore (Billerica, USA). ReNcell VM are derived from the ventral midbrain of a 10-week-old fetus and immortalized by retroviral v-Myc transduction. They were cultivated in culture vessels at 37°C, 20% O_2 and 5% CO_2. To grow cells in adherent monolayers, vessels need to be coated with Laminin (stock 1mg/ml) (Trevigen, Gaithersburg, USA), diluted 1:100 in ice-cold DMEM:F12 (Invitrogen) for at least one hour at 37 °C.

Cells were grown until 80% confluency and harvested by incubating at 37°C with Trypsin/Benzonase until detaching. Reaction was stopped by the addition of Trypsininhibitor/Benzonase. The suspension was centrifuged for 5min at 1500rpm. The supernatant was discharged and the cell pellet was resuspended in fresh medium with growth factors. A defined cell number was seeded in fresh culture vessels.

Differentiation of cells was induced by the removal of growth factors by changing the medium.

2.2.1.2 Cultivation of ST14A cells

ST14A cells, a conditionally immortalized rat embryonic (E14) striatal progenitor cell line with a temperature-dependent expression of an immortalizing oncogene, have been described before (Cattaneo et al., 1998). The cells were obtained from embryonic day 14 (E14) rat striatum and were transfected with the temperature-sensitive mutant tsA58U19 of the SV40 large T antigen (Weinelt et al., 2003) Cells were cultured in flasks and chamber slides at 33°C for proliferation and 39°C for differentiation, respectively.

2.2.1.3 Determination of cell number

The number of cells was counted using a CASY cell counter (Roche, Germany). On passing the measurement capillary a determined amount of electrolyte solution is displaced by the cells. This results in a change resistance which is a dimension for the cell volume. This technique allows separate viable from dead cells as dead cells are smaller and therefore have a different resistance.
A defined volume of cell suspension was added to 10ml CASYton and measured with the appropriate calibrated program.

2.2.1.4 Transfection of cells

For the transfection of ReNcell VM with either overexpression or reporter gene plasmids the Nucleofection System (Lonza, Cologne, Germany) together with Nucleofector Kit V was used. Briefly, $2x10^6$ cells were resuspended in Nucleofection solution and transfected with 2µg plasmid and program X-001 according to the manufacturer's instructions. Subsequently, cells were plated in laminin-coated culture vessels and grown up to 80% confluence. Cells were then differentiated by the withdrawal of growth factors. Small molecules were added at the start of differentiation, diluted in differentiation medium.

2.2.1.5 Cell proliferation assay

Cells were seeded at a defined cell number and proliferated for 24h. Then the medium was changed and substances or DMSO as a control were added at an appropriate concentration (timepoint 0h). The cell number and viability were measured every day by using the CASY cell counter system. For each time point a triplicate was determined.

2.2.1.6 WST-1 assay

Cell viability was measured using the colorimetric assay WST-1 (Roche, Penzberg, Germany) that determines the enzyme activity of mitochondrial dehydrogenases, which

cleave a tetrazolium substrate resulting in colored formazan. The enzyme activity correlates with the metabolic activity of viable cells (Hipper and Isenberg, 2000). 10000 cells per well were seeded in laminin-coated 96-well plates in proliferation medium. Drugs were applicated 24h after cell seeding in either proliferation or differentiation medium. Cell viability was assessed after 24h and 48h by adding 10µl WST-1 reagent (10% of the total volume) per well and incubation of the plates for 2h at 37°C and 5% CO_2. The optical density at 450nm wavelength was determined using genios microplate reader (Tecan, Crailsheim, Germany). 650nm was used as reference wavelength. Per condition, six wells were measured.

2.2.2 Molecularbiological methods

2.2.2.1 RNA-isolation

RNA was isolated by using the RNeasy Plus Mini Kit (Qiagen, Hilden). Briefly, cells were harvested (2.2.1.1), centrifuged for 5min at 300xg. The pellet was resuspended in 400µl RLT plus buffer. $5x10^6$ cells were used to isolate RNA according to the manufacturer's instructions. RNA was eluted in 2x 30µl RNAse free water. The quantity and quality of total RNA was determined by spectroscopy (2.2.2.2) and denaturating agarose gel electrophoresis (2.2.2.3). Samples were stored at -80°C.

2.2.2.2 Photometric RNA concentration measurement

The quantity and quality of total RNA were analyzed by measuring the absorption at 260 and 280nm using a spectrophotometer (Nanodrop, ThermoScientific, Wilmington, USA) and denaturating agarose gel electrophoresis (2.2.2.3). A 260/280nm ratio of 1.8-2.0 indicated high qualtity RNA.

2.2.2.3 RNA agarose gel electrophoresis

For the integrity check of RNA samples, RNA was separated using formalydehyde gels. Gels were generated by adding 10ml of 10x FA gel buffer and 90ml RNase free H2O to 1.2g agarose. The mixture was heated to melt agarose and then cooled to 65°C. 1.8 ml of 37% (12.3M) formaldehyde and 1µl of a 10mg/ml ethidium bromide stock solution were added, mixed and poured onto the gel support. The gel was equilibrated in 1x formaldehyde gel running buffer for 30min. RNA samples were mixed with the appropriate volume of 2x RNA loading dye (MBI Fermentas, St.-Leon-Roth Germany). The gels were run at 100V and photographed using UV gel documentation system (Herolab, Wiesloch, Germany) in combination with a digital camera sytem (C-5050; Olympus, Japan). Size estimation of RNA fragments was done using marker RiboRuler (MBI Fermentas). Sharp bands of 28S/18S rRNA with an intensity ratio of about 2:1 indicated intact RNA.

2.2.2.4 TaqMan quantitative real-time PCR

A customized 384-well format TaqMan Array (Applied Biosystems Inc, Foster, USA) was used for gene mRNA quantification. The first cDNA strand was synthesized with 400ng of total RNA as template using High Capacity cDNA kit (Applied Biosystems) according to the manufacturer's instruction. Each slot was loaded verb missing equivalently with cDNA transcribed from 200ng RNA. Samples were run in duplicate on a 7900HT real-time PCR system (ABI).
Cycling parameters were as followed:

Enzyme activation:	50°C	2min
Initial denaturation:	95°C	20sec
Denaturation:	95°C	1sec
Annealing and extension:	60°C	20sec

The threshold line was set automatically by SDS 2.3 software and the threshold cycle (CT) values were determined by the threshold line crossing the amplification curve. Target transcription abundance was quantified using the delta-delta C_T method. PCR data were normalized to GAPDH and expressed as relative changes compared to timepoint 0h.

2.2.2.5 Luciferase reporter gene assay

For the analysis of TCF-dependent transcription ReNcell VM cells were transfected with p12xSuperTOPFlash vector or p8xFOPflash and renilla luciferase vector phRL-TK (kindly provided by Randall T. Moon) with the Nucleofection system (Lonza, Cologne, Germany), according to the manufacturer's recommendation. As a positive control, cells were co-transfected with the vector pCAGGS-S33Y containing a stabilized form of β-catenin. In this vector, the phosphorylation site Ser33 is replaced by Tyrosine, resulting in a constitutively active form (Kim et al., 2000). Subsequently cells were plated in laminin-coated 48-well plates and cultured as described above. 24h after transfection, the proliferation conditions were changed to differentiation conditions. At the start of differentiation, drugs were added at indicated concentrations. The activity of the firefly luciferase and constitutively expressed Renilla luciferase was measured using the luminometer LB 9508 (Berthold, Bad Wildbad, Germany) and the Dual Luciferase Assay Kit (Promega, Madison, USA).

2.2.3 Protein analysis

2.2.3.1 Protein determination

The amount of protein in ReNcell VM cells extracts was determined by the use of the BCA protein assay reagent kit (Thermo Scientific, Rockford, USA). Cells were lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (Roche) and centrifuged for 5min at 15000rpm. The supernatant was used for further experiments. 200µl BCA reaction solution were added to 10µl of total cell protein and incubated for 1h at 37°C.

The absorbance was measured at 570nm with a plate photometer (Tecan, Crailsheim, Germany).

2.2.3.2 ELISA

The stabilization of β-catenin was measured using the human total β-catenin ELISA DuoSet IC system (R&D Systems, Wiesbaden, Germany) according to the manufacturer's recommendation. 15µg of total cell lysate were used. The optical density was measured with a plate photometer (Tecan) at 450nm with a wavelength correction at 570nm.

Cells were differentiated 24h after seeding for 2h. The substances were added at indicated concentrations at the start of differentiation.

2.2.3.3 GSK-3β activity assay

IC_{50} of new synthesized compound AB199 to GSK-3β was determined by a luminometric GSK-3β activity assay. This method has been described by Baki et al. (2007) and gives IC_{50} values comparable to a radioactive detection. Briefly, compounds were tested in different concentrations diluted in assay buffer containing final concentrations of: 4mM MOPS pH 7.2; 0.4mM EDTA; 1mM EGTA; 2.5mM β-glycerophosphate; 4mM $MgCl_2$; 40µM BSA; 0.05mM DTT. 4µl of diluted compounds were added to 25µM pGS-2 peptide substrate (Millipore, Billerica, USA), 20ng recombinant GSK-3β (R&D systems) and 1µM ATP (Cell Signaling, Boston, USA) to a total assay volume of 40µl. The enzymatic reaction was stopped after 30min of incubation at 30°C by adding 40µl KinaseGlo (Promega, Madison, USA). The luminometric signal was allowed to stabilise for 10min and then measured with a Glomax® 96 Microplate Luminometer (Promega).

2.2.3.4 GSK-3β *in vitro* kinase assay

ReNcell VM cells were differentiated at 80% confluence for 2h. The test substances were added at the start of differentiation at indicated concentrations.

Cells were lysed in RIPA buffer, supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) and centrifuged for 5min at 15000rpm. The supernatant was used for further experiments. Immunoprecipitation of GSK-3β was performed with a specific mouse monoclonal anti GSK-3β [G8] antibody (Abcam, Cambridge, UK) with 5µg/sample for 2h at 4°C. The bound protein was precipitated with Protein A/G-Plus agarose-beads (10µl beads per sample; Santa Cruz Biotechnology, Santa Cruz, USA). GSK-3β kinase activity was measured in a reaction mixture containing final concentrations of: 4mM MOPS pH 7.2; 0.4mM EDTA; 1mM EGTA; 2.5mM β-glycerophosphate; 4mM $MgCl_2$; 40µM BSA; 0.05mM DTT. 10µg/sample pGS-2 peptide substrate (Millipore, Billerica, USA) was used.

The assay was initiated by the addition of a mixture of unlabelled ATP (final concentration: 50 µM) and [γ-^{32}P] ATP (Hartmann Analytic, Braunschweig, Germany; 4µCi per sample). Following 20min incubation at 37°C the assay was stopped by centrifugation at 10,000*g* for 1min and spotting the supernatant onto phosphocellulose discs. The filter mats were washed once in 1% acetic acid and 3 times in H_2O. Afterwards, pGS-2 peptide-associated γ-^{32}P radioactivity bound to the phosphocellulose was quantified by Cerenkov counting.

2.2.3.5 Immunocytochemistry

ReNcell VM cells were cultured and differentiated as above. Shortly, ReNcell VM were seeded on cover slips and proliferated for 3 days and subsequently differentiated by the withdrawal of growth factors. Cells were fixed with 4% PFA in 0.1% PBS for 15min, at time points 0h and 3 days, whereas 0h was the time point of the induction of differentiation. Afterwards the cells were treated with blocking solution containing 0.4% TritonX-100 for 30 min at room temperature. The primary antibody for βIII-tubulin (Santa Cruz, 1:500 mouse monoclonal) was incubated for 1hr as well as the secondary antibody (Alexa Fluor 568 goat anti rabbit, 1:1000, Molecular Probes). Probes were sealed with mounting

medium containing DAPI (1.5µg/ml, Vector Labs, USA) as a marker for cell nuclei. Microscopic analysis was performed using the Keyence Biozero system (Keyence, Neu-Isenburg, Germany).

ST14A cells were cultured in 8-well chamber-slides under proliferation conditions. With the change to differentiation conditions, a media change was performed and the drugs were added at indicated concentrations to the media. At time points 0h and 6h, the cells were fixed with 4% paraformaldehyde in 0.1% PBS for 10min. After washing, unspecific binding sides were blocked with PBS containing 0.3% Triton X-100 and 5% goat serum for 30min. Cultures were incubated with the primary antibody for 30 min at room temperature. β-catenin specific antibody (mouse mAb, Santa Cruz, Heidelberg, Germany) was diluted 1:200 in PBS with 1% goat serum. The slides were washed with PBS and then incubated with the secondary antibody (Alexa Fluor 568 goat anti-mouse antibody, diluted 1:100 in PBS with 1% goat serum) for 30min at room temperature. After washing with PBS, the cells were covered with mounting medium (Vector Labs, Burlingame, CA, USA) containing DAPI (1.5µg/ml) for nuclear staining. Microscopic analysis was performed using the Keyence Biozero microscope (Keyence).

2.2.3.6 Flow cytometry

The influence of GSK-3β inhibitors on neuronal differentiation of ReNcell VM cells was determined by FACS analysis. Cells were stained for βIII-tubulin and the total number of positive cells was measured. For this purpose cells were cultured in 6-well plates up to 80% confluency and subsequently differentiated for 3 days. The medium supplemented with substances was changed every 24h. For FACS analysis, cells were trypsinized and centrifuged at 100xg at RT for 5min, washed with PBS without Ca^{2+} and Mg^{2+} and fixed with 1% PFA in PBS for 15min. Cells were then resuspended in washing buffer (PBS + 0.5 % BSA + 0.02 % Na-azide) and stored at 4°C in the dark. For the staining cells were centrifuged and resuspended in saponin buffer (PBS + 0.03% saponin + 0.5% BSA + 0.02% Na-azide) containing βIII-tubulin antibody (Santa Cruz, 1:100 mouse monoclonal) and incubated for 2h at RT. Afterwards the cells were washed and incubated with the secondary antibody (Alexa Fluor 647 goat-anti-mouse, 1:1000, Molecular Probes) for 1h

in saponin buffer. Cells were washed twice with saponin buffer and resuspended in wash buffer for analysis. Measurement was done using FACSCalibur (BectonDickinson, San Jose, USA) in combination with Cell Quest Pro software.

2.2.3.7 Apoptosis assay

ReNCell VM cells were cultured under proliferation conditions up to 80% confluence. Then cells were differentiated for up to 12h in the presence of 3µM SB-216763, AB199 or the equivalent amount of DMSO. Cells were harvested and stained with Annexin/PI using the FITC Annexin V Apoptosis Detection Kit I (BectonDickinson) according to the manufacturer's recommendation. Measurement was done using FACSCalibur (BectonDickinson) in combination with Cell Quest Pro software.

2.2.4 Statistical analysis

All results are shown as mean ± SEM of data, whereat N indicates the number of biological repeats and n the number of technical repeats. Statistical analysis was performed with a Student's t-test. $p < 0.05$ was considered to indicate statistically significance using Excel (Microsoft, USA) and Prism5 (GraphPad Prism Inc., USA).

3 Results

3.1 Screening for a new GSK-3β inhibitor

In order to develop a screening system for novel GSK-3β inhibitors in hNPCs, potential targets/readouts to investigate their influence on Wnt/β-catenin signaling and its effect on hNPC proliferation and differentiation were tested regarding their applicability for a multi-level screening system. Potential targets were chosen by downstream-events of GSK-3β inhibition.

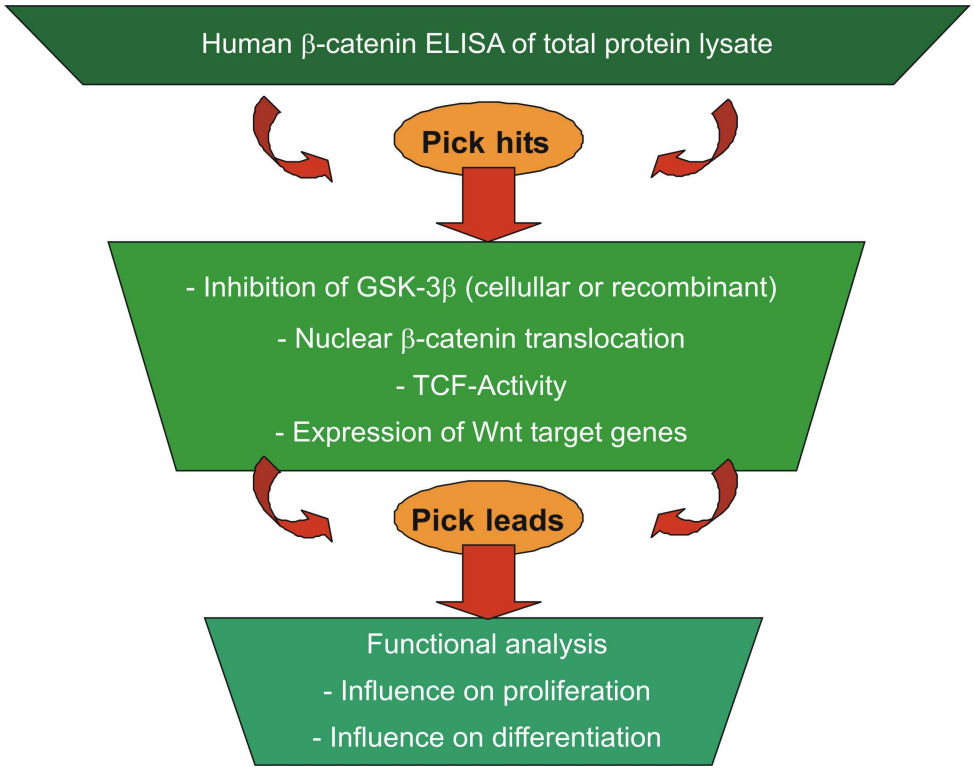

Figure 4: Potential readouts for a screening system for a novel GSK-3β-inhibitor.

3.1.1 Time-dependent accumulation of β-catenin

As the phosphorylation and degradation of cytosolic β-catenin is mediated by GSK-3β as a member of the β-catenin degradation complex, the accumulation of β-catenin was an attractive target for screening approaches.

To evaluate which timepoint is best to investigate the influence of novel small molecules on β-catenin accumulation, the expression of β-catenin was analyzed over the time. ReNcell VM cells were seeded at a defined number, cultivated up to 80% confluence and then differentiated by the withdrawal of growth factors as described in 2.2.1.1. With the start of differentiation, the known GSK-3β inhibitors Kenpaullone and SB-216763 were added at indicated concentrations. Cells were harvested and proteins were isolated at defined time points (2.2.3.1). The amount of total β-catenin was determined by an ELISA specific for β-catenin (2.2.3.2).

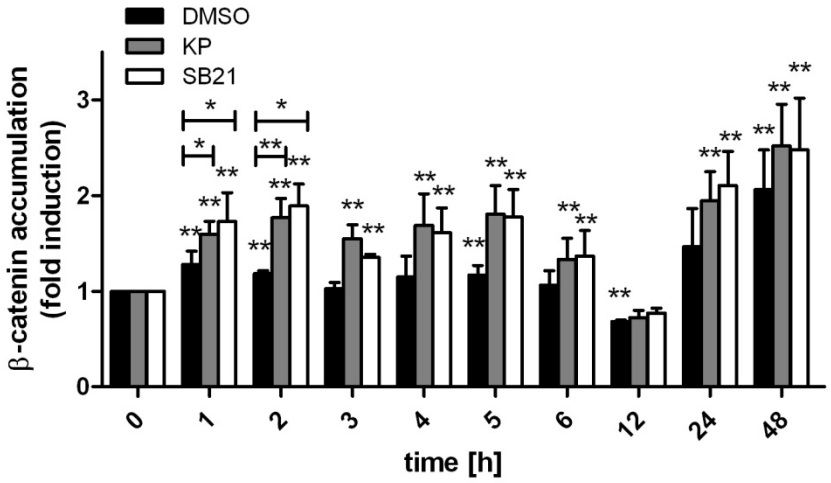

Figure 5: Total β-catenin in ReNcell VM cells after treatment with Kenpaullone and SB-216763. Cells were cultured for 0-48h under differentiation conditions in the presence of known GSK-3β inhibitors. Concentrations were 1µM for Kenpaullone (KP) and 3µM for SB-216763 (SB21). β-catenin level was evaluated with a β-catenin specific ELISA. The level of β-catenin increases at the start of differentiation. This effect can even be intensified by the treatment with GSK-3 inhibitors. Data were normalized to 0h differentiation and represent mean +/- SEM (N=3-7, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at *p< 0.05 or **p<0.01.

Differentiation of ReNcell VM results in an increase of the amount of total β-catenin already after 1h. This rise of the β-catenin level compared to proliferating cells is constant up to 6h. After 12h a decrease can be seen (Fig.5). The highest amount of total β-catenin can be detected after 48h. Treatment of cells with SB-216763 or Kenpaullone displayed a similar expression profile, whereas the increase of β-catenin levels was stronger compared to control cells. This difference between control and drug-treated cells was significant after 1h and 2h of treatment, albeit the amount of total β-catenin under the influence of SB-216763 was slightly higher after 2h incubation (Fig.5). Therefore, time point 2h was chosen for further screening approaches.

3.1.2 Screening for novel small molecules as potential GSK-3β inhibitors

191 novel small molecules were tested regarding their ability to increase β-catenin accumulation, but only substance groups AB and GK are presented in detail. The tested substances were provided by the Leibniz-Institute for Catalysis.

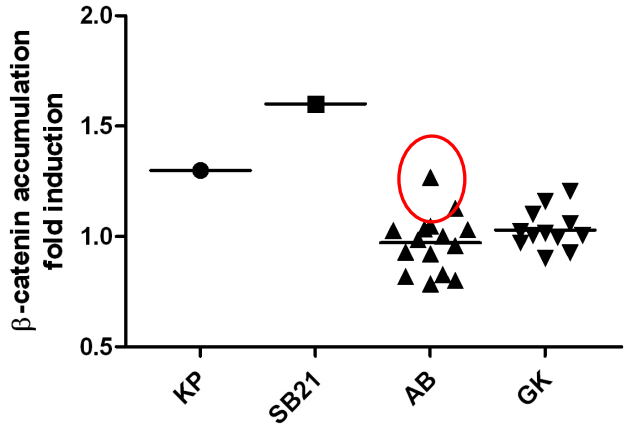

Figure 6: Total β-catenin in ReNcell VM cells after treatment with novel small molecules.
Cells were cultured for 2h under differentiation conditions in the presence of known GSK-3β inhibitors or new synthesized small molecules. β-catenin level was evaluated with a β-catenin specific ELISA. Concentrations were 1µM for Kenpaullone (KP) and 3µM for SB-216763 (SB21) and substances, respectively. Data were normalized to DMSO control and represent mean +/- SEM (N=3-4, each done in triplicates).

As shown in Fig.6, none of the newly designed small molecules (AB-substances, Fig.7) was able to increase β-catenin accumulation in the same range as SB-216763. Several substances reached the same level as Kenpaullone. One substance which resulted in a similar action as Kenpaullone was AB199, highlighted in red. To investigate if any modifications of AB199 could improve its effects, 12 derivatives of AB199 (GK-substances, Fig.8) were tested. None of the modifications showed an improvement in β-catenin accumulation. Therefore, AB199 alone was characterized in a detailed way, regarding its influence on the Wnt/β-catenin pathway as well as functional assays regarding proliferation and differentiation of human neural progenitor cells.

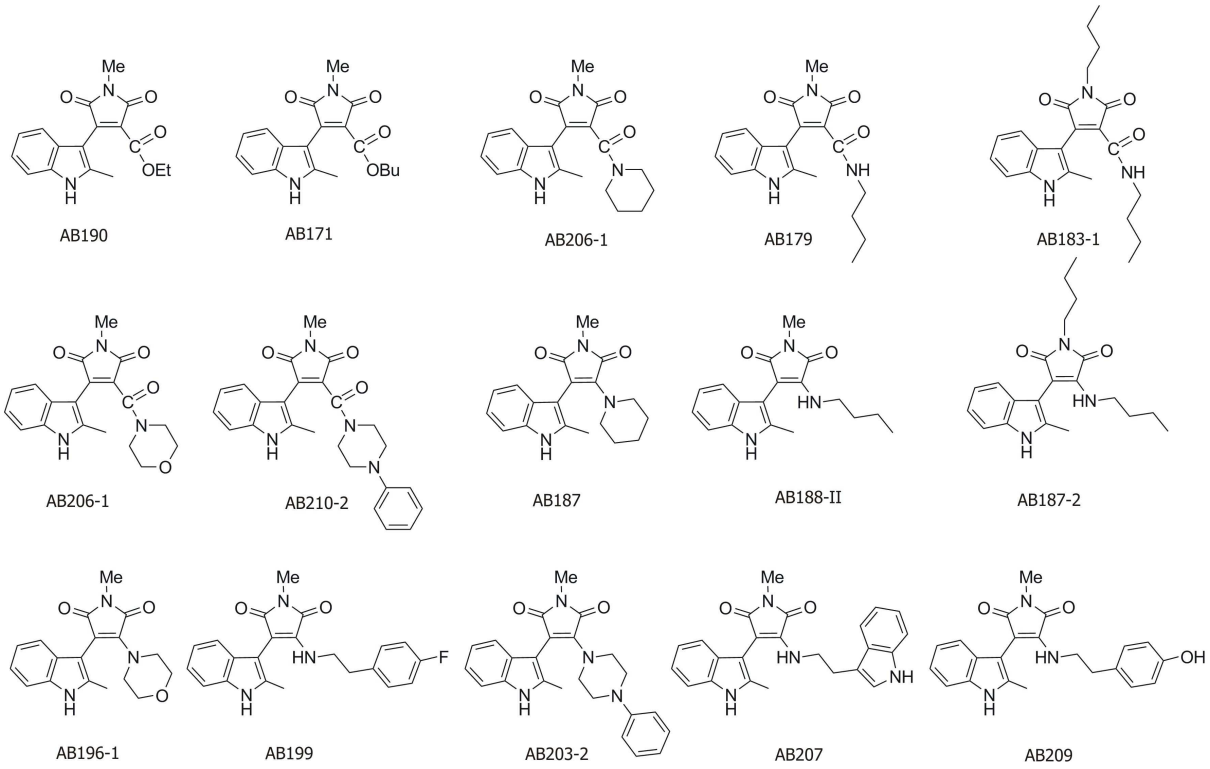

Figure 7: Chemical structures of various non-symmetrically substituted indolylmaleimides tested for biological activity.

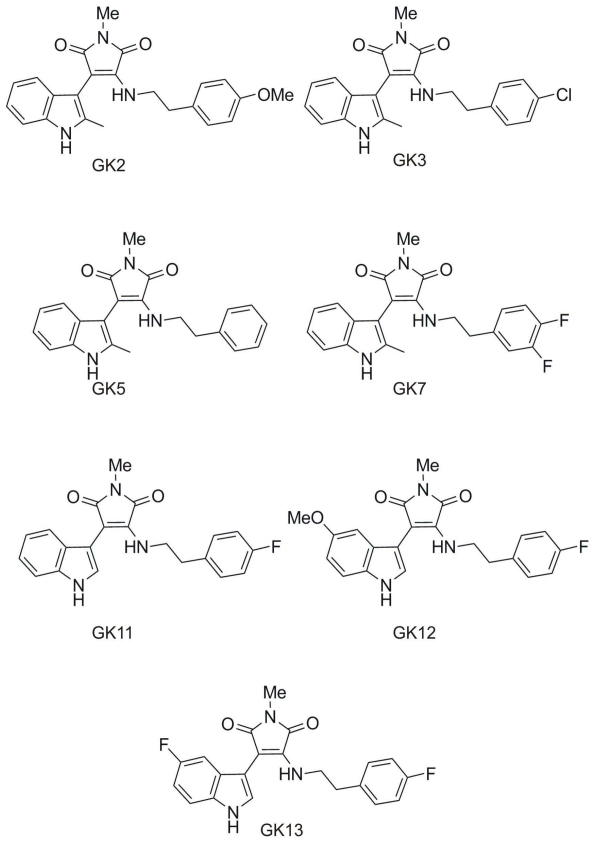

Figure 8: Small chemical library based on AB199.

3.1.3 Characterization of potential GSK-3β inhibitor AB199

3.1.3.1 Determination of the ideal working concentration of AB199 in hNPC

To determine the concentration of AB199 with the optimal biological response in hNPC, seven concentrations of AB199 from 0.1µM to 10µM were measured. First, the influence of different concentrations of SB-216763 and AB199 on cell metabolic activity as a cellular toxicity assay was tested. WST is a tetrazolium salt which is cleaved by mitochondrial dehydrogenases into formazan (Berridge et al, 1996). The amount of formed formazan correlates with the metabolic activity of the cells and can be measured photometrically. Drugs were diluted in either proliferation or differentiation medium and applied to the cells. Medium with fresh drugs was changed every 24h, and cell metabolic activity was measured 24h and 48h after initial drug application (2.2.1.6). Concentrations from 1µM to 3µM were tested. It could be observed that none of the tested concentrations had a toxic effect on hNPCs, whereas the treatment with SB-216763 for 24h increased cell metabolic activity (Fig.9a). The same effect could be observed in a concentration-dependent manner for AB199, but this effect was not significant (Fig. 9c). Any significant changes for SB-216367 under differentiation conditions are of statistic origin (Fig. 9b). AB199 slightly diminishes cell metabolic activity after 24h under differentiation conditions (Fig. 9d).

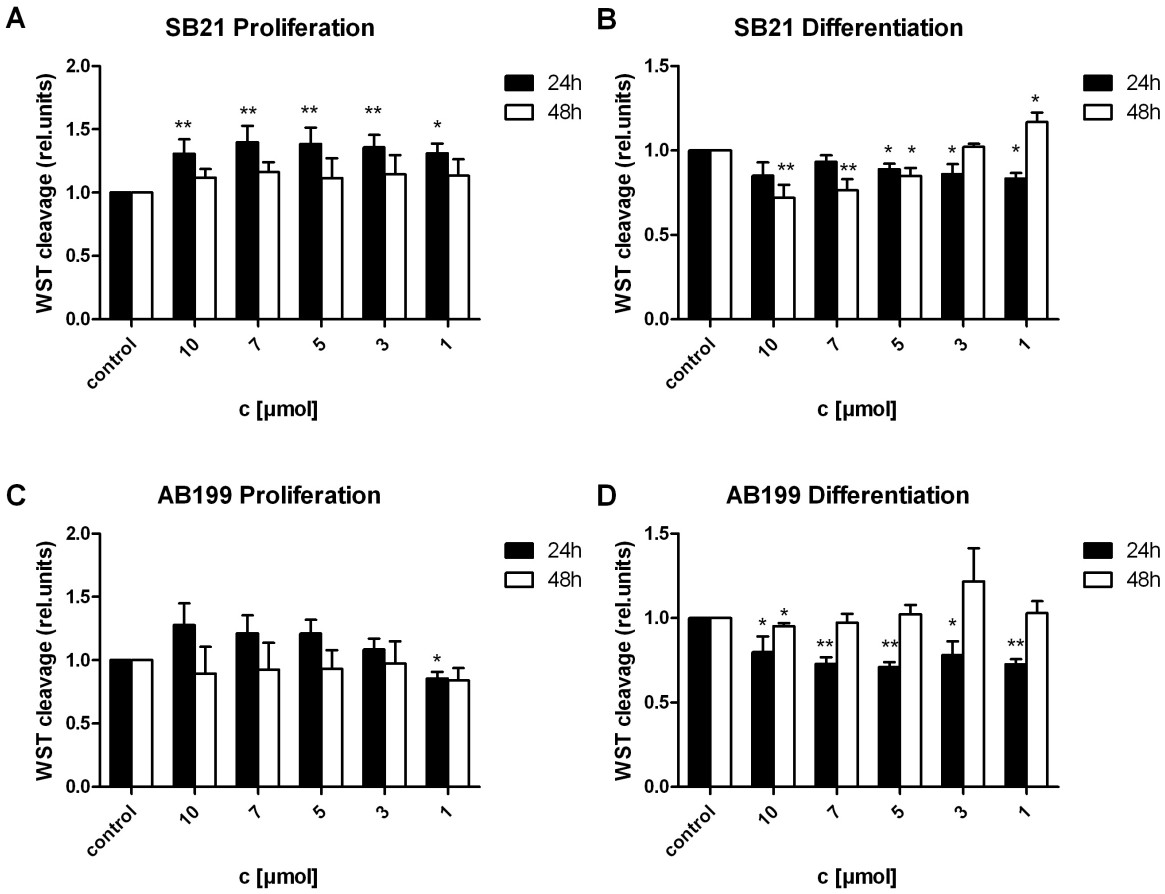

Figure 9: Metabolic activity in proliferating and differentiating hNPC under the influence of SB-216763 and AB199.
Cells were cultivated under proliferation or differentiation conditions in the presence of SB-216763 (SB21) or AB199 from 1-10µM. After 24h and 48h, cell metabolic activity was measured with WST-1 assay. None of the tested concentrations of both drugs had a toxic effect on cell metabolic activity of hNPC. Data were normalized to DMSO control cells and represent mean +/- SEM (N=4, each done in sextuplets). Values were significantly different between drug treated and DMSO treated control cells at *p< 0.05 or **p<0.01.

As none of the tested concentrations was revealed to be toxic, concentrations of AB199 from 0.1 to 10µM were investigated regarding their ability to accumulate β-catenin. Therefore an ELISA specific for human total β-catenin was used (2.2.3.2). At concentrations from 0.1µM to 3µM a concentration-dependent increase of β-catenin accumulation from equal to 1.3-fold compared to control cells could be observed (Fig. 10a). This effect rapidly decreased when concentrations of AB199 from 5-10µM were used, indicating a tight dose-window. An EC_{50}-concentration of 3.8µM was calculated, whereat a bell-shaped dose-response curve was monitored. Therefore the use of 3µM for further experiments was reasonable.

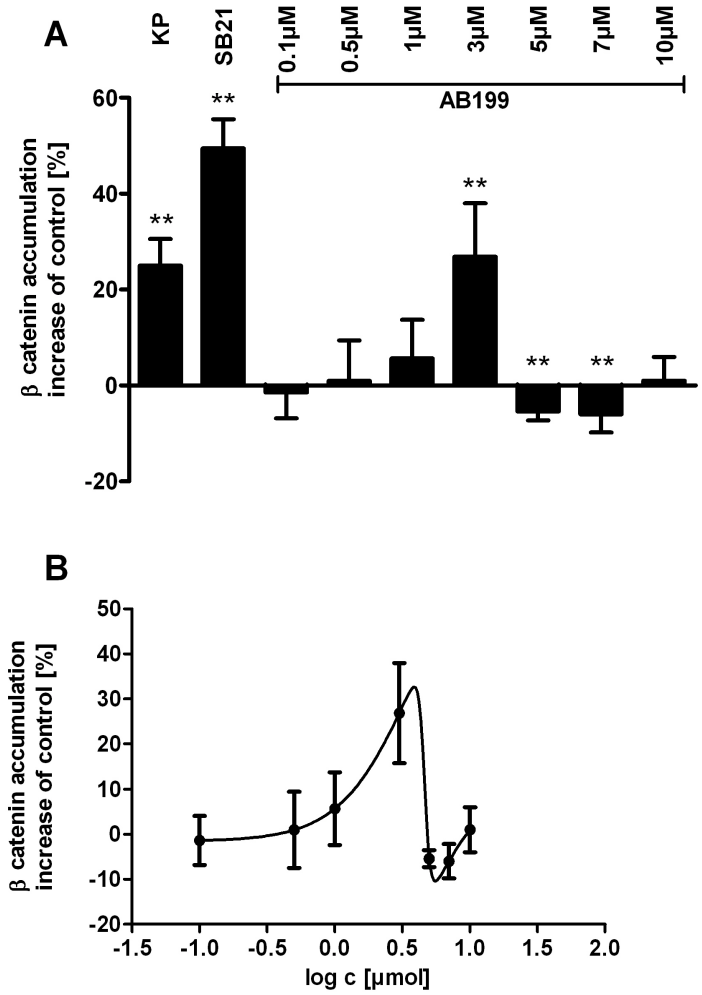

Figure 10: Total β-catenin in ReNcell VM after treatment with different concentrations of AB199.

A: AB199 augmented β-catenin accumulation significantly at a concentration of 3µM. Lower or higher concentration had no further effect. B: EC_{50} determination for β-catenin accumulation. Cells were cultured for 2h under differentiation conditions in the presence of AB199. Concentrations were 1µM for Kenpaullone (KP), 3 µM for SB-216763 (SB21) and variable for AB199. Data were normalized to DMSO control and represent mean ± SEM (N = 3–10, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at *p <0.05 or **p <0.01.

Furthermore it was of interest to examine whether combinations of AB199 with SB-216367 could even ameliorate the single observed effect. Thus, SB-216763 at a concentration of 3µM was combined with various concentrations of AB199, ranging from 0.1-10µM and were tested regarding their potency to accumulate β-catenin.

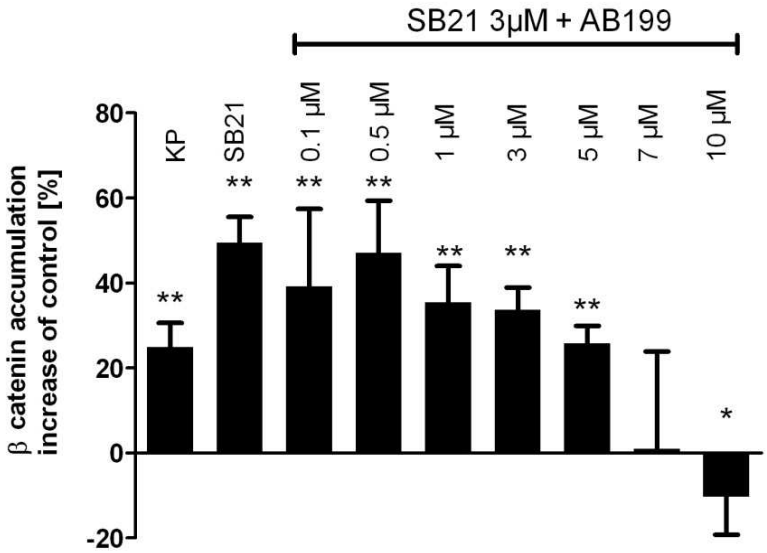

Figure 11: The combination of SB-216763 and AB199 resulted in no additive effect to SB-216763. Cells were cultured for 2 h under differentiation conditions in the presence of AB199. Concentrations were 1µM for Kenpaullone (KP), 3µM for SB-216763 (SB21) and variable for AB199. Data were normalized to DMSO control and represent mean ± SEM (N = 3–10, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at *p <0.05 or **p <0.01.

As shown in Fig.11 no additive effect to SB-216763 was observed. Interestingly, the combination of 3µM SB-216763 and 10µM of AB199 depleted the β-catenin level in a significant way, whereas 3µM SB-216763 together with lower concentrations of AB199 (0.1–5µM) showed no effect.

3.1.3.2 Inhibition of GSK-3β by AB199

As AB199 showed to accumulate β-catenin in hNPC, its mechanism of action was of interest. Due to its close structural similarity to SB-216763, it is most likely that AB199 inhibits GSK-3β. In order to prove this assumption an *in vitro* kinase assay. hNPC were treated for 2h with either SB-216763 or AB199 under differentiation conditions. Cells were then lysed and total protein was isolated. GSK-3β was immunoprecipitated by a specific antibody. Remaining kinase activity was measured by phosphorylation of GSK-3β substrate and ATP-consumption (2.2.3.4).

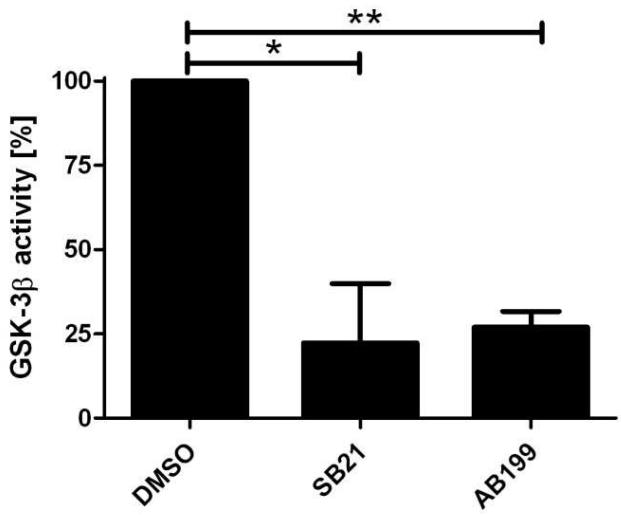

Figure 12: Inhibition of GSK-3β by SB-216763 and AB199.
Cells were cultured for 2h under differentiation conditions in the presence of AB199. Concentrations were 1µM for Kenpaullone (KP), 3µM for SB-216763 (SB21) and variable for AB199. GSK-3β was immunoprecipitated and remaining activity was measured by the turnover of ATP. The known GSK-3β inhibitor downregulated GSK-3β activity to 22 ± 18%. AB199 acted in the same range and inhibited GSK-3β to a remaining activity of 27 ± 5%. Data represent mean ± SEM (N = 3). Values were significantly different between drug treated cells and DMSO treated control cells at *p <0.05 and **p <0.01.

These results suggest that AB199 acts as a GSK-3β inhibitor. To determine its sensitivity, a GSK-3β activity assay was performed. Here, GSK-3β was not immunoprecipitated out of hNPC but a recombinant enzyme was used. This assay makes it possible to investigate the direct effect on GSK-3β and does not take into consideration any effect of other cellular activities on the enzyme.

An IC_{50} for the inhibition of GSK-3β was obtained by treating recombinant GSK-3β with different concentrations of either SB-216763 or AB199. Remaining activity was measured as a luminometric signal which is inversely correlated with kinase activity (2.2.3.3).

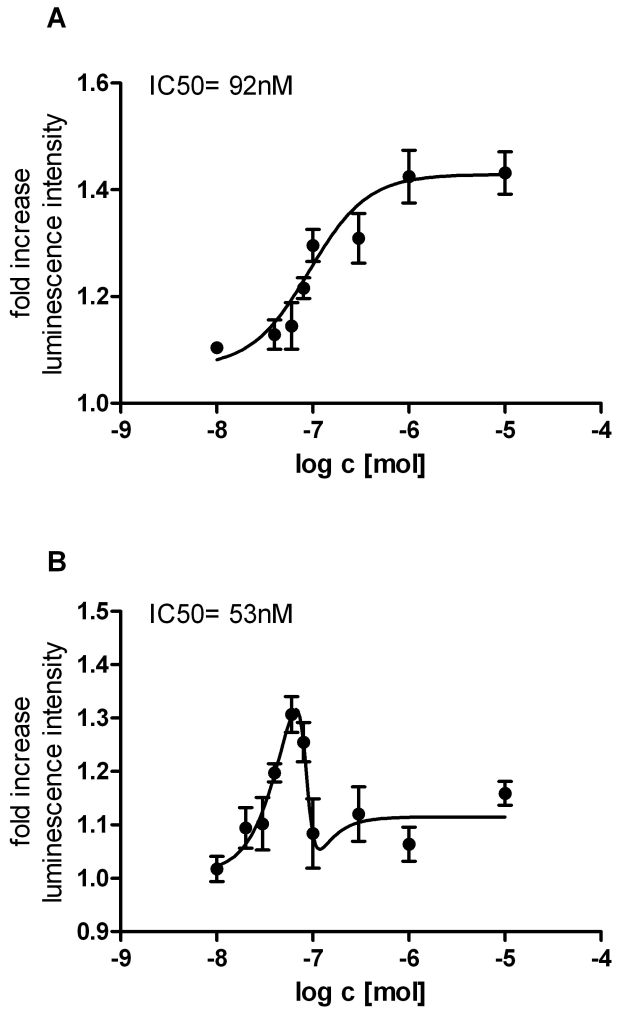

Figure 13: Inhibition of GSK-3β by SB-216763 and AB199.
IC_{50} value for GSK-3β inhibition by SB-216763 (A) and AB199 (B) were determined in a kinase activity assay. The luminescence signal is inversely correlated with kinase activity. The evaluation revealed IC_{50} values of 92nM for SB-216763 and 53nM for AB199. Data were normalized to control and represent mean ± SEM (n = 3–8).

The experiments revealed that both substances inhibit GSK-3β in a dose-dependent matter. For SB-216763, an IC_{50} value of 92nm was calculated (Fig. 13a). The IC_{50} value for AB199 was slightly lower with 53 nM (Fig. 13b). Interestingly, a bell-shaped dose-response curve was observed for AB199, whereas the dose-response curve displayed a sigmoidal run. The same bell-shaped run under AB199-conditiong was observed for beta-catenin accumulation (Fig. 10b)

3.2 Influence of novel GSK-3β inhibitor AB199 on Wnt/β-catenin signaling

GSK-3β is the key enzyme of the Wnt/β-catenin pathway. It forms, together with APC, CK1 and Axin, the β-catenin degradation complex (MacDonald et al., 2009), which triggers the phosphorylation and ubiquitination of β-catenin, resulting in activation of the Wnt-pathway. Inhibition of GSK-3β results in the accumulation of β-catenin and the activation of Wnt downstream targets.

3.2.1 Nuclear accumulation of β-catenin mediated by AB199

To visualize nuclear β-catenin, a cell line derived from rat striatum (ST14A) was treated with either SB-216763 or AB199 and immunocytochemically stained for β-catenin. ST14A cells have been described previously as a model for visualizing nuclear accumulation of β-catenin, whereas ReNcell VM in our hands did not show a clear accumulation of β-catenin which was most likely due to the growth pattern of the cells (Fig. 14).

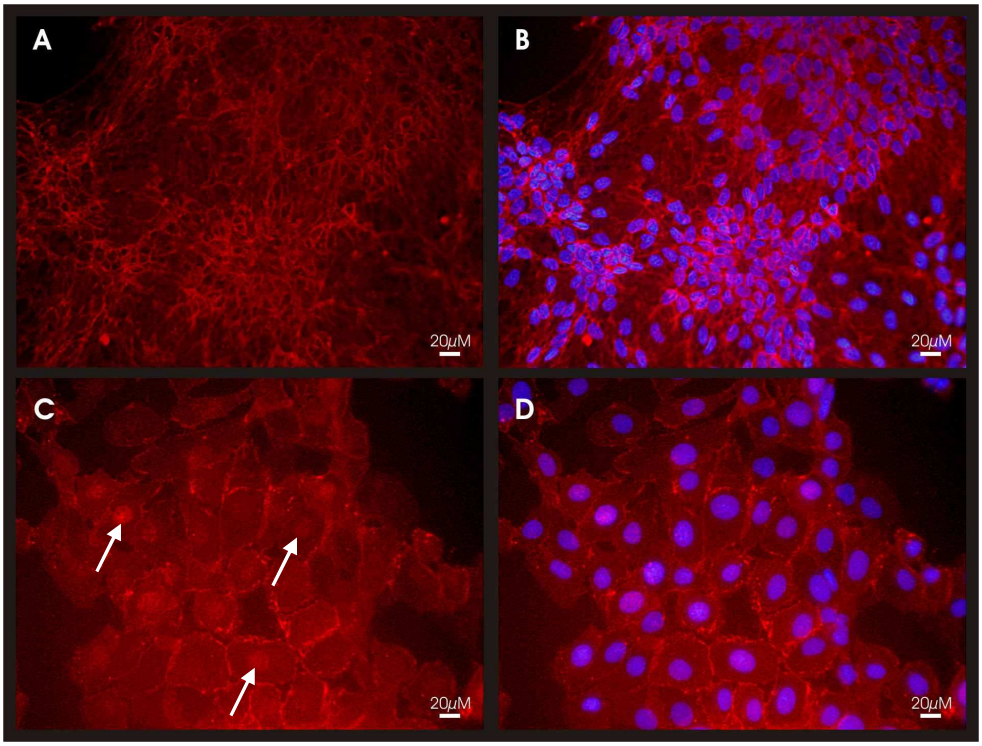

Figure 14: Nuclear β-catenin translocation in ReNcell VM cells and ST14A cells.
hNPCs (A+B) and ST14A cells (C+D) were differentiated for 1d and stained for β-catenin (red) and DAPI (blue). hNPCS (A+B) show a dense growth pattern, cell boarders cannot be identified. ST14A cells (C+D) are larger and display a flat morphology. Cells are clearly separated from each other, single cells can be seen. Arrows indicate nuclear β-catenin accumulation. The scale bar indicates a size of 20µm.

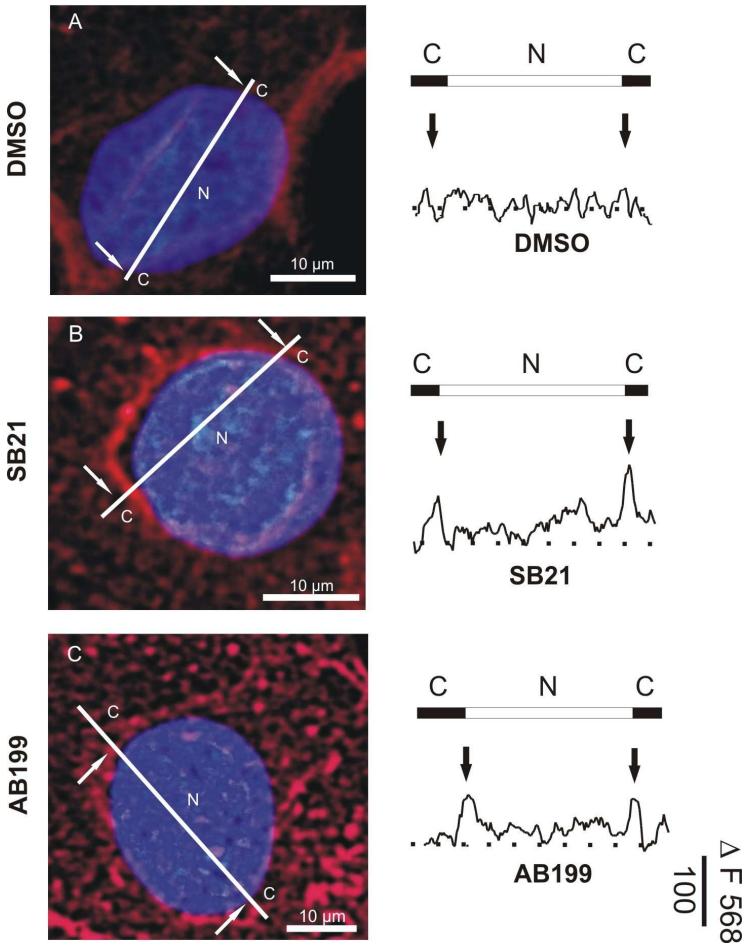

Figure 15: GSK-3β inhibitors elevate nuclear β-catenin accumulation after 6 h of differentiation. ST14A cells were stained for β-catenin (red) and DAPI (blue). Left panel: (A) In DMSO treated cells no β-catenin accumulation was observed. In contrast, in SB-216763 (SB21) (B) or AB199 (C) treated cells, an accumulation of β-catenin around the nucleus was observed. Right panel: The peri-nuclear accumulation is also visible in a line-scan detecting the fluorescence intensity (F568) of the cytosol (C) and nucleus (N). Arrows indicate the peri-nuclear accumulation of β-catenin. The scale bar indicates a size of 10µm.

When cells were differentiated for 6h, cells showed no nuclear accumulation of β-catenin (Fig. 15a). Treatment with SB-216763 led to a clear enrichment of β-catenin (Fig. 15b). This was confirmed with a linescan, which quantifies the fluorescence intensity. AB199 was able to imitate this effect, showing that AB199 not only inhibits GSK-3 and increases β-catenin accumulation but also triggers its translocation to the nucleus (Fig. 15c).

3.2.2 Induction of TCF-activity

After β-catenin has been translocated to the nucleus, it replaces Groucho and interacts with the TCF/LEF transcription factors and activates the transcription of Wnt target genes. The activity of those transcription factors can be measured with a reporter gene assay. hNPCs were transfected with TOPFlash (containing 12 TCF binding sites), FOPFlash (a control vector which contains mutant TCF binding sites). Previous experiments revealed that a co-transfection with pCAGGS-S33Y was necessary, a vector which includes a mutated, stabilized form of β-catenin, to detect TCF-activity in these cells. It seems that a certain threshold of β-catenin has to be overcome. As the accumulation of β-catenin had already shown to be time-dependent, several time points were analyzed regarding their TCF-activity.

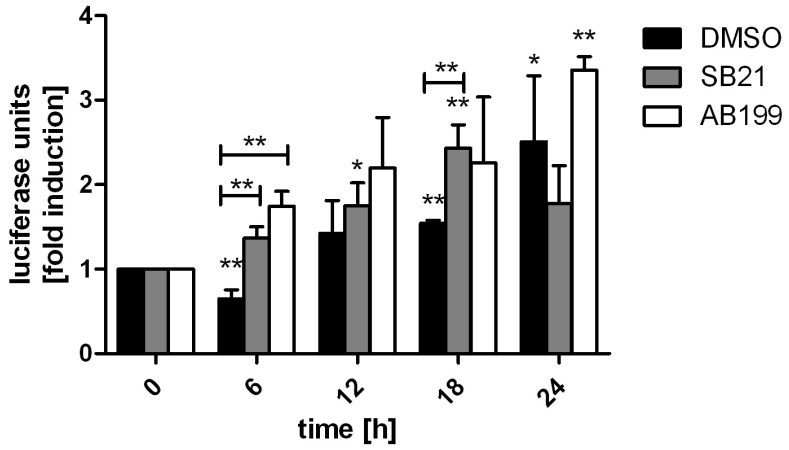

Figure 16: TCF-activity of hNPC after treatment with SB-216763 and AB199.
Cells were transfected with TOP/FOP plasmids and co-transfected with pCAGGS-S33Y (TCF reporter gene assay SuperTOPflash). 24 hours after transfection, conditions were changed from proliferation to differentiation with the addition of small molecule at a concentration of 3µM. TCF-activity was measured after 0-24h of differentiation. Ratio TOP/FOP was normalized to control cells. Data represent mean ± SEM (N = 5, each done in triplicates). Values were significantly different between drug treated cells and DMSO treated control cells at *p <0.05 and *p<0.01.

The induction of differentiation of hNPCs by the withdrawal of growth factors led to decrease of TCF-activity after 6h, but then to a time-dependent increase (Fig. 16, black column). This suggests that Wnt, together with the time-dependent accumulation β-catenin after start of differentiation, that Wnt/β-catenin signaling is involved in the differentiation process of hNPCs. SB-216763 influenced TCF-activity in a positive way,

compared to control cells, resulting in a peak after 18h of differentiation (2.3 fold compared to 0h) followed by a decrease (Fig. 16, grey column). AB199 can even boost this effect, which is clearest after 24h (1.5 fold by SB-216763 and 3.2 fold by AB199, compared to 0h).

3.2.3 Gene expression-profile of components of the Wnt/β-catenin pathway

As it has been successfully shown that GSK-3β inhibition results in an upregulation of TCF-activity, it was of interest to examine how GSK-3β inhibition manipulates the gene expression of components of the Wnt/β-catenin signaling pathway or Wnt target genes.
hNPCs were differentiated for up to 4 days in the presence of either DMSO (control), SB-216763 or AB199. At defined time points, cells were harvested and total RNA was isolated (2.2.2.1). Isolated RNA was transcribed into cDNA and then analyzed with a customized TaqMan array (2.2.2.4).

3.2.3.1 Expression of Wnt receptors

It has been previously shown that hNPCs can respond to Wnt/β-catenin signaling as they express endogenously Wnt receptors and respond to ligand-binding (Mazemondet et al., in preparation). Native differentiation was characterized and revealed an increase in expression of Wnt receptors. Therefore it was of interest to investigate the effect of GSK-3β inhibitors on the expression of Wnt receptors.

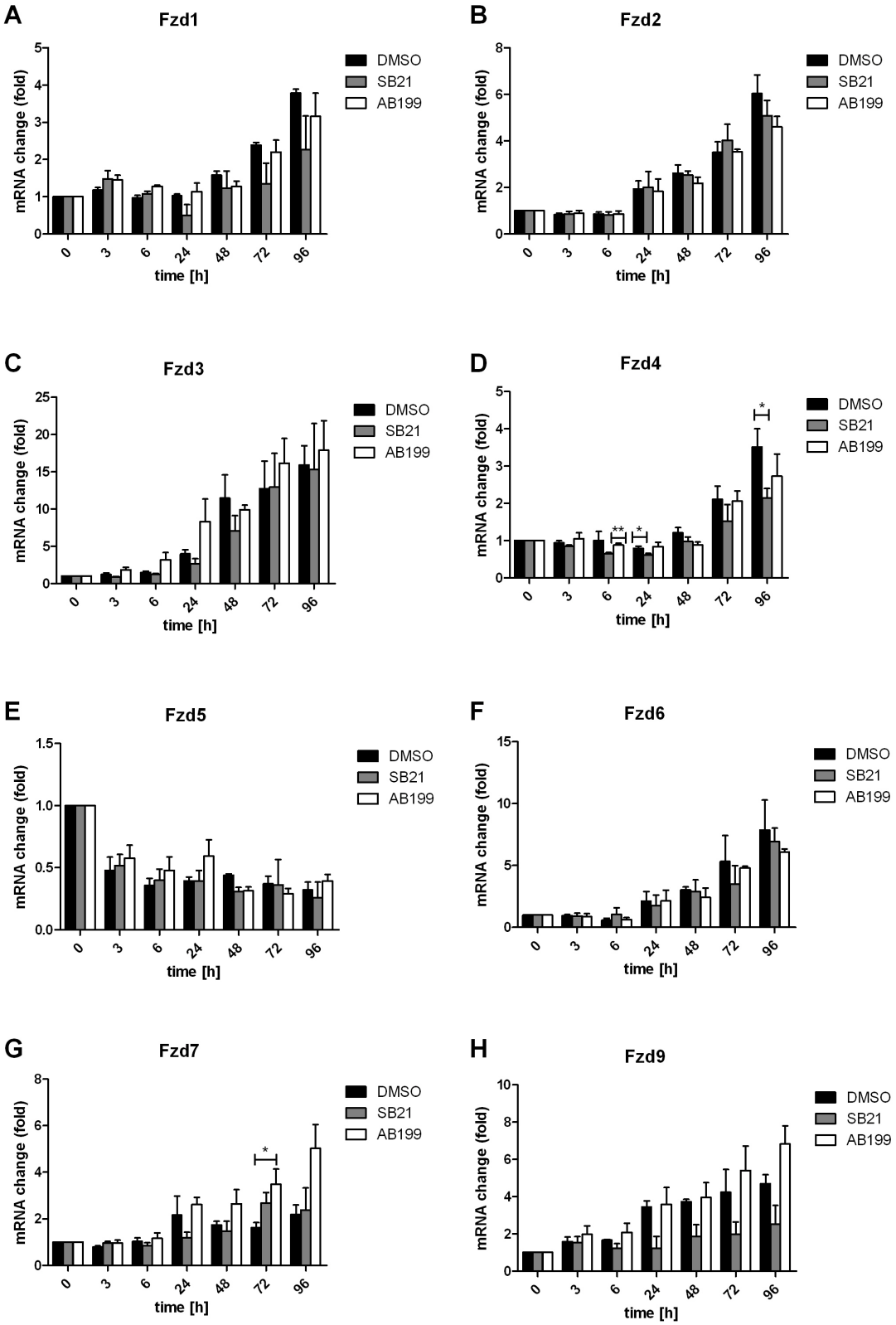

Figure 17: Expression of Wnt signaling receptors.
hNPCs were differentiated up to 96h under the influence of 3µM SB-216763 (SB21), 3µM AB199 or the equivalent amount of DMSO. Cells were harvested at indicated time points and mRNA levels of Frizzled receptors (Fzd) were detected by TaqMan quantitative real-time PCR. Values were normalized to GAPDH. Data were normalized to 0h differentiation and represent mean +/- SEM (N=4, each done in duplicates). Values were significantly different at *p< 0.05 or **p<0.01.

All Frizzled-Rezeptors could be detected in hNPCs, whereas Fzd8 and Fzd10 were expressed in a very low manner and thus could not be quantified (Tab. 2). All Frizzled-Receptors were upregulated during differentiation, except Fzd5 which displayed decreased expression. Treatment with GSK-3β inhibitors resulted in no severe changes in expression profiles (Fig. 17), whereat AB199 showed a tendency to increase Fzd7-expression. Fzd9-expression was downregulated under the influence of SB-216367, but this effect was not significant. This effect could not be counterfeited by AB199 (Fig. 17h).

3.2.3.2 Expression of Wnt signaling co-receptors

In addition to the determination of the expression of several Wnt-receptors, the expression profiles of different co-receptors were analyzed. Like the expression of Wnt-receptors, the expression of co-receptors increased at the start of differentiation over the time.

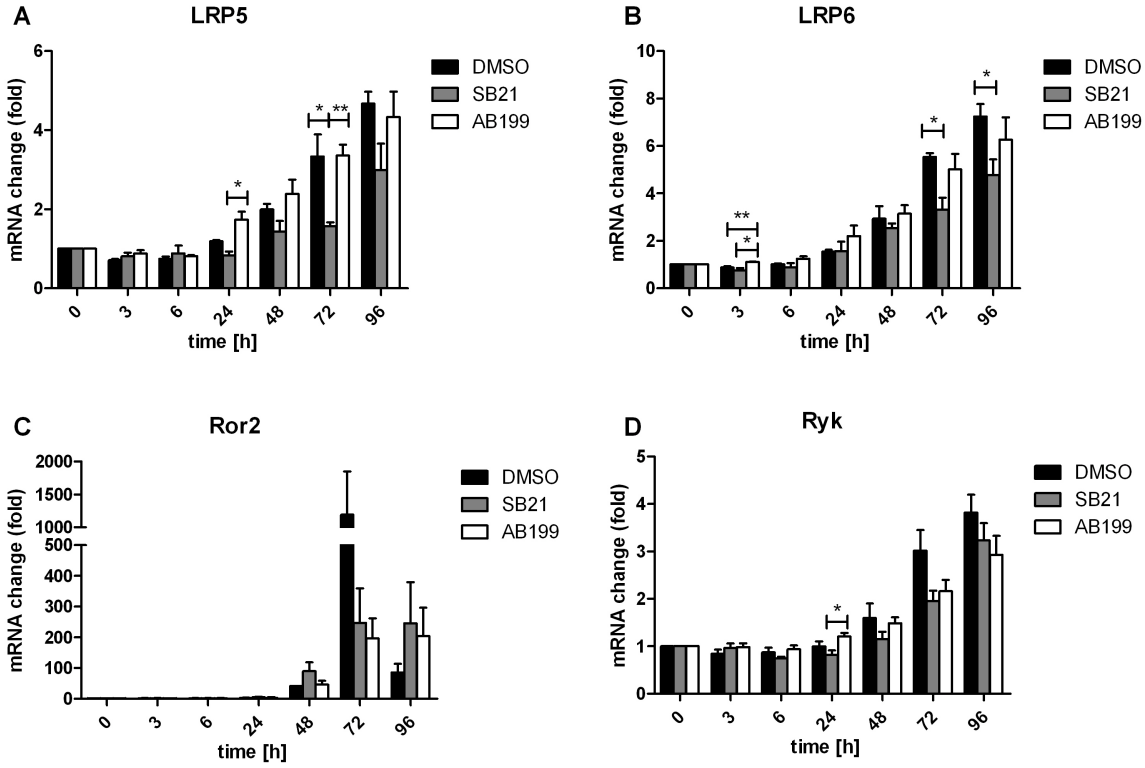

Figure 18: Expression of Wnt signaling co-receptors and alternative receptors.
hNPCs were differentiated up to 96h under the influence of 3µM SB-216763 (SB21), 3µM AB199 or the equivalent amount of DMSO. Cells were harvested at indicated time points and mRNA levels of Wnt-receptors were detected by TaqMan quantitative real-time PCR. Values were normalized to GAPDH. Data were normalized to 0h differentiation and represent mean +/- SEM (N=4, each done in dublettes). Values were significantly different at *p< 0.05 or **p<0.01.

For LRP5 and 6, some changes following small molecule-treatment could be observed. Expression of LRP5 was significantly downregulated after 72h under the influence of SB-216763, compared to control or AB199-treated cells (Fig. 18a+b). The same goes for LRP6 after 72h and 96h. Expression of Ror2 and Ryk was not significantly influenced by the presence of SB-216763 or AB199 (Fig. 18c+d), whereat expression Ror2 shows a massive increase during late differentiation, starting from time point 48h to 96h .

3.2.3.3 Expression of Wnt ligands

As only some slight changes in the expression of Wnt-receptors caused by the exposition of hNPCs during differentiation were monitored, we wanted to know whether they might have an influence on the expression of Wnt-ligands. Out of 19 Wnts, only Wnt5a, 5b,

Wnt7a and Wnt10b could be detected in a quantifiable manner (Fig. 19). All the others were either not detectable or expressed at a very low level (Tab. 2).

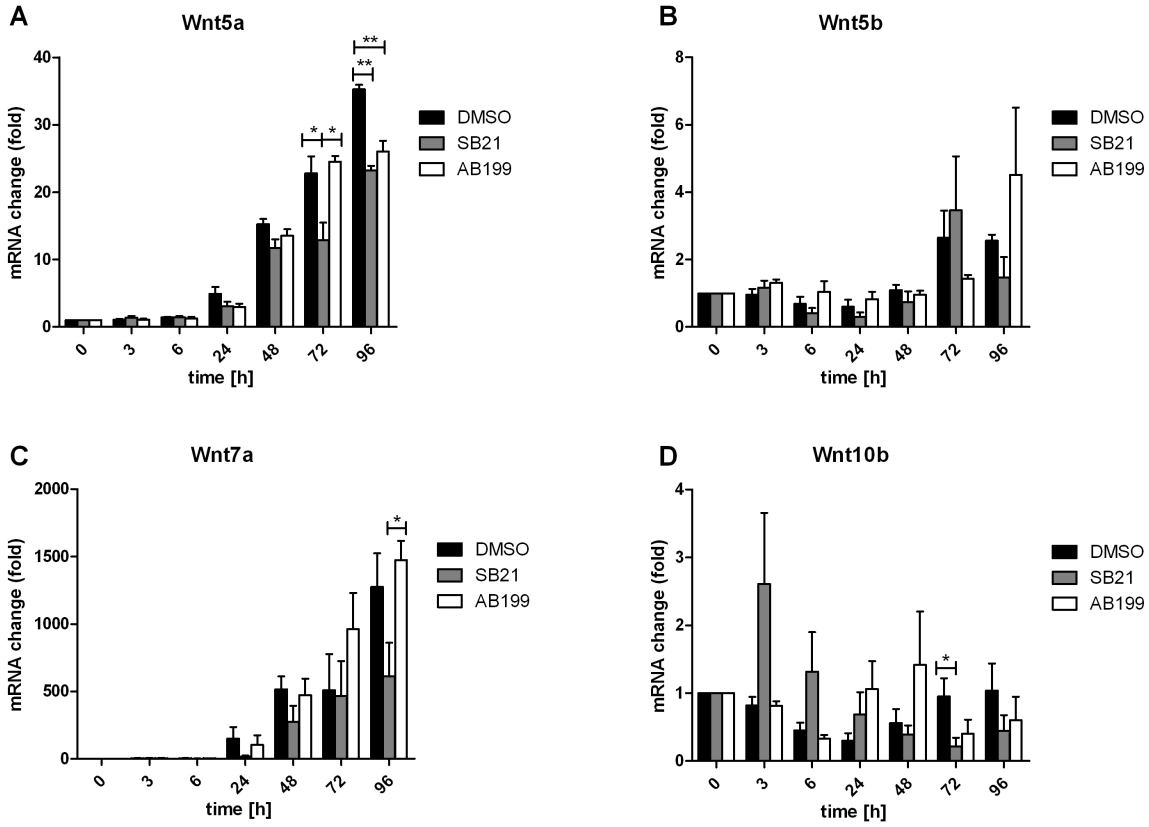

Figure 19: Expression of Wnt ligands.
hNPCs were differentiated up to 96h under the influence of 3µM SB-216763 (SB21), 3µM AB199 or the equivalent amount of DMSO. Cells were harvested at indicated time points and mRNA levels of Wnt-ligands were detected by TaqMan quantitative real-time PCR. Values were normalized to GAPDH. Data were normalized to 0h differentiation and represent mean +/- SEM (N=4, each done in duplicates). Values were significantly different at *p< 0.05 or **p<0.01.

The expression of Wnt5a (Fig. 19a) and Wnt7a (Fig. 19b) increased during differentiation. Whereas Wnt5a was upregulated up to about 35-fold after 96h, the increase of Wnt7a expression was about 1800-fold. Treatment with SB-216763 decreased Wnt5a-expression after 72h in a significant way compared to control or AB-199 treated cells. After 96h, both SB-216763 and AB199 treated cells showed a reduced expression of Wnt5a compared to control cells (Fig. 19a).

3.2.3.4 Expression of Wnt signaling modulators and target genes

An activation of Wnt/β-catenin signaling results in the transcription of Wnt target genes. Our experiments have shown that the differentiation of hNPCs induces the activation of Wnt/β-catenin signaling, which could also be manipulated by novel GSK-3 inhibitors. Therefore the expression of different Wnt target genes or modulators was evaluated.

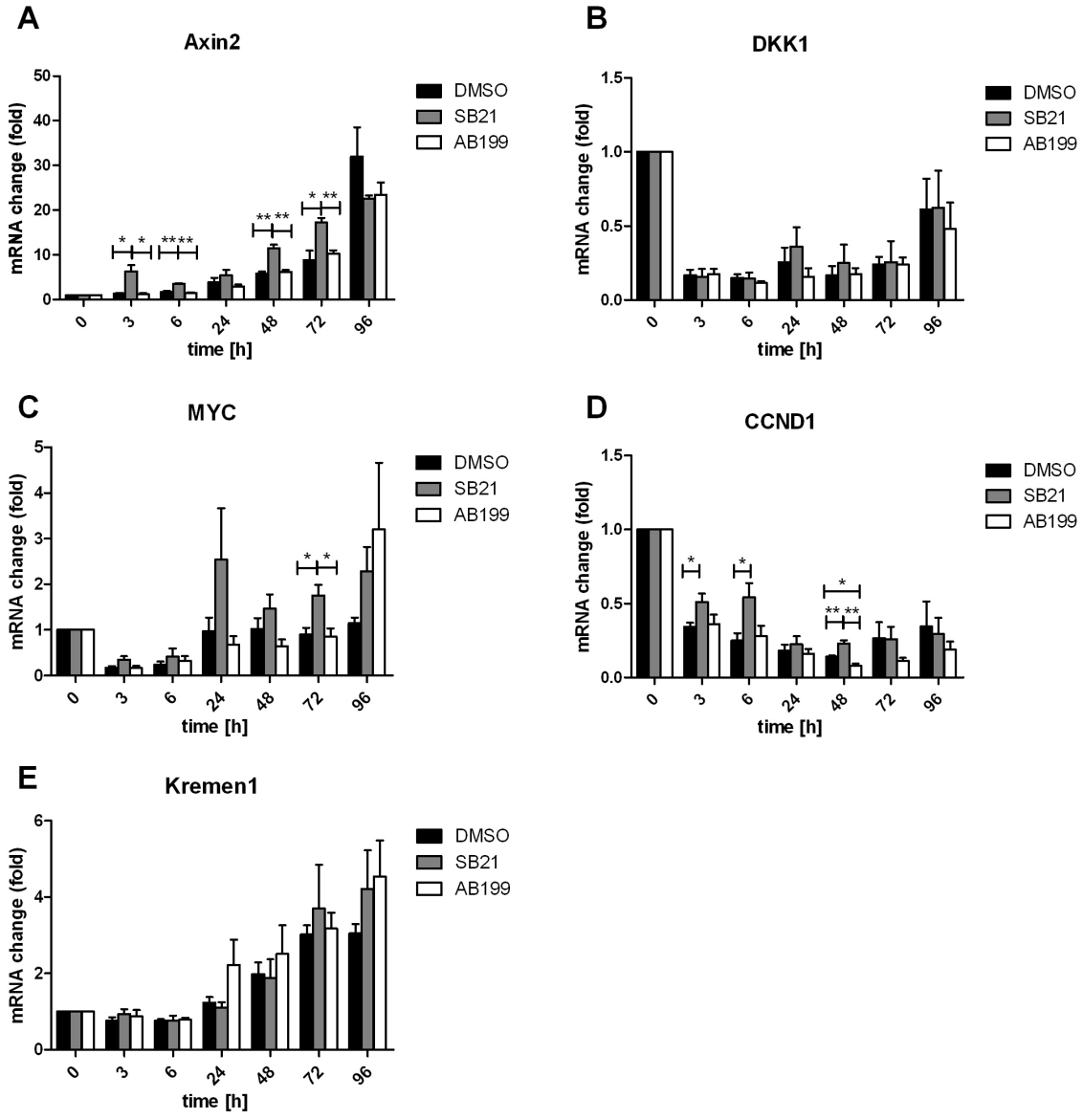

Figure 20: Expression of Wnt signaling modulators and target genes.
hNPCs were differentiated up to 96h under the influence of 3µM SB-216763 (SB21), 3µM AB199 or the equivalent amount of DMSO. Cells were harvested at indicated time points and mRNA level were detected by TaqMan quantitative real-time PCR. Values were normalized to GAPDH. Data were normalized to 0h differentiation and represent mean +/- SEM (N=4, each done in duplicates). Values were significantly different at *p< 0.05 or **p<0.01.

Differentiation of hNPCs induced the expression of Axin2 and Kremen1 (Fig. 20a+e), whereas Dkk1 (Dickkopf1) and CCDN1 (Cyclin D1) were downregulated (Fig. 20b+d). SB-216763 was not able to increase the expression of Axin2 significantly after different time points compared to control cells, which could not be observed for AB199 (Fig. 20a). In addition, SB-216763 changed the expression profile slightly for CCDN1. The downregulation of CCDN1 was not that strong in the presence of SB-216763 compared to control cells or AB199-treated cells (Fig. 20d).

In conclusion, differentiation of hNPCs had a strong impact on the expression of Wnt-related genes. Interestingly, the inhibiton of GSK-3β had only slight effects on the expression profile, only some genes, like Fzd7, Fzd9 or Axin2 were affected by the presence of GSK-3β inhibitors. Any observed modulating-effects were caused by the treatment of hNPCs with SB-216763, the expression profile of AB199-treated hNPCs was more similar to control cells.

Time point of upregulation	Maxiumum increase 1-5-fold	Maxiumum increase 5-10-fold	Maxiumum increase >10-fold
0-6 hours			
6 hours-2 days	c-Myc	Fzd2 Fzd7 Fzd9 LRP5 LRP6 Ryk Axin2	Fzd3 WNT5A WNT7A Ror2
>2 days	Fzd1 Fzd4 Wnt5b Kremen1	Fz6	
Time point of downregulation	**Maxiumum decrease 1-5-fold**	**Maxiumum decrease 5-10-fold**	**Maxiumum decrease >10-fold**
0-6 hours	Fzd5 Dkk1 Cyclin D1		
6 hours-2 days	WNT10B		
>2 days			
Very Low expression		**Not detected**	
LEF1 Fz8 Fz10 KREMEN2 WNT3 WNT4 WNT7B WNT9B WNT11 Rspo2		WNT1 WNT2 WNT2B WNT3A WNT6 WNT8A WNT8B WNT9A WNT10A WNT16 Rspo3	

Table 2: Summary of Taq Man qRT-PCR analysis of gene expression regulation.
Genes were ordered arranged according to their time point and extend of up/downregulation.

3.3 Influence on cellular processes by novel GSK-3β inhibitors

Wnt/β-catenin signaling is connected to several processes such as differentiation, proliferation, cell death, cell polarity, self- renewal and morphogenic movements (Moon et al, 2004). As it had been successfully demonstrated that AB199 acts as a GSK-3β inhibitor and possesses the ability to activate and support endogenous Wnt signaling

during differentiation, the next step was to investigate how this manipulation affects proliferation and differentiation of hNPCs.

3.3.1 Novel GSK-3β inhibitors inhibit cell proliferation of hNPCs

To investigate whether or not cell proliferation was influenced by the presence of GSK-3β inhibitors, cells were seeded at a defined number and proliferated for 24h. Then GSK-3β inhibitors were added to the medium and cells were cultivated under proliferation conditions. Every 24h, fresh medium containing drugs was provided and the cell number was counted (2.2.1.5).

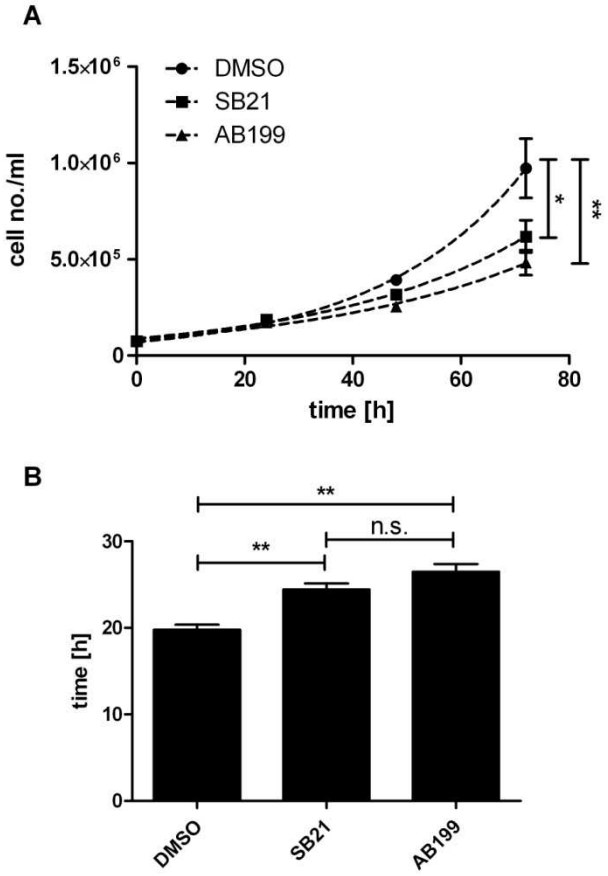

Figure 21: Cell proliferation of hNPCS is decelerated after treatment with GSK-3β inhibitors.
(A) Growth curve of treated hNPCs. Cells were seeded at a defined cell density and were cultivated in the presence of DMSO, SB-216763 or AB199. The cell number was determined every 24h. (B) Doubling time of hNPCs. Doubling times were calculated with the data from growth curves in Figure 21A. The conditioning of proliferating cells with SB-216763 or AB199 resulted in a significant increase of the cell doubling time. Concentrations were 3µM for SB-216763 (SB21) and AB199. After 72h of treatment, SB-216763 and AB199 significantly reduced the cell proliferation.

Data represent mean ± SEM (N = 4, each done in triplicates). Values were significantly different between drug treated cells and DMSO treated control cells at *p <0.05 and **p <0.01.

hNPCs showed an exponential growth curve and a doubling time of 19.8 ± 0.6h was calculated. In contrast, treatment of the cells with SB-216763 and AB199 increased the cell doubling time significantly to 24.4 ± 0.7h and 26.5 ± 0.9h, respectively (Fig. 21a). The numbers of SB-216763 and AB199 treated cells were significantly reduced after 72 h compared to the number of DMSO treated control cells (Fig. 21b). This effect seemed not be cytotoxic as cell viability was not influenced (data not shown).

As the conditioning of hNPCs with GSK-3β inhibitors lead to decreased cell proliferation the question arose whether these results are connected to the accumulation of β-catenin. Do substances which increase β-catenin accumulation also decelerate cell proliferation of hNPCs and maybe the other way around or is this a phenomenon which is specific to SB-216763 and AB199? To verify this hypothesis, different small molecules which either increased or decreased β-catenin accumulation were chosen to evaluate their influence on cell proliferation. Substances that ameliorated β-catenin accumulation were: PDA228-1, PDA 228-3 and e993-0255, substances that depleted β-catenin accumulation were: AB209, PDA167, g288-0012.

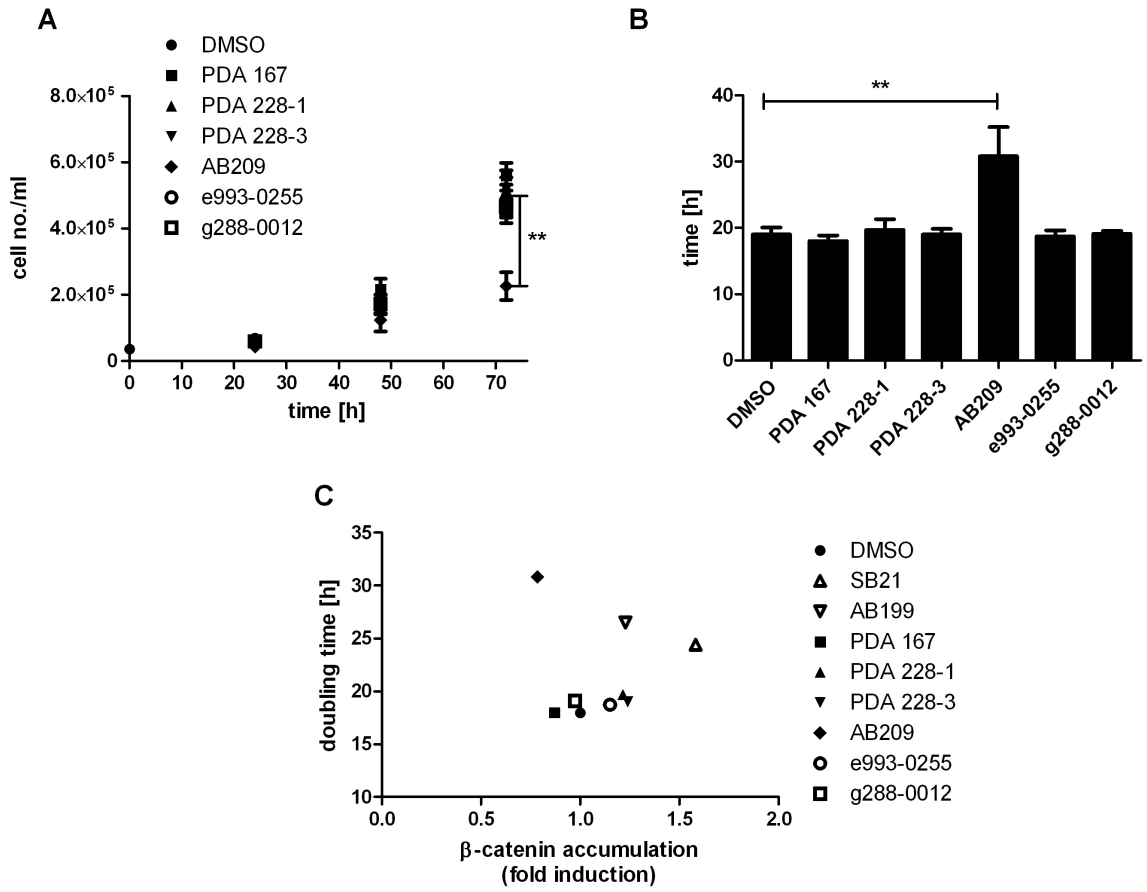

Figure 22: Relation between cell doubling time and β-catenin accumulation in hNPCs.
(A) Growth curve of treated hNPCs. Cells were seeded at a defined cell density and were cultivated in the presence of DMSO and different small molecules. The cell number was determined every 24h. (B) Doubling time of hNPCs. Doubling times were calculated with the data from growth curves in Figure 22A. (C) Relation between cell doubling time and β-catenin accumulation. Data represent mean +/- SEM (N=4, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at *p< 0.05 or **p<0.01.

None of the tested substances seemed to affect cell proliferation as there was no significant difference in the cell number or in the cell doubling time (Fig. 22a and b). Only AB209 significantly decelerated cell proliferation and decreased the cell doubling time (Fig. 22a and b). AB209 is of high structural similarity to AB199.

Fig 22c shows the relation between β-catenin accumulation and cell proliferation. No relation between β-catenin accumulation and cell proliferation was observed.

3.3.2 Neuronal differentiation of hNPCs is influenced by GSK-3β inhibition

As it was demonstrated that proliferation of hNPCs is decelerated by GSK-3β inhibition, it was investigated how the inhibition of GSK-3β influences neuronal differentiation of hNPCs.

First the gene expression of neuronal markers Tuj1 and Map2 were analyzed. hNPCs were differentiated for up to 4 days in the presence of either DMSO (control), SB-216763 or AB199. At defined time points, cells were harvested and total RNA was isolated (2.2.2.1). Isolated RNA was transcribed into cDNA and then analyzed with a customized TaqMan array (2.2.2.4).

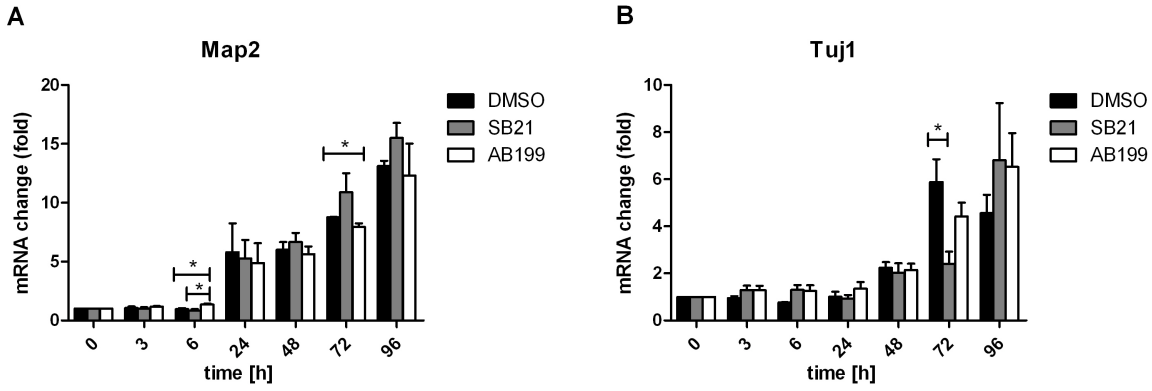

Figure 23: Expression of neuronal markers in differentiating hNPCs.
hNPCs were differentiated up to 96h under the influence of 3µM SB-216763 (SB21), 3µM AB199 or the equivalent amount of DMSO. Cells were harvested at indicated time points and mRNA level of Map2 and Tubb3 were detected by TaqMan quantitative real-time PCR. Values were normalized to GAPDH. Data were normalized to 0h differentiation and represent mean +/- SEM (N=4, each done in dublettes). Values were significantly different at *p< 0.05 or **p<0.01.

Fig. 23 shows that the gene expression of both markers increases at the start of differentiation. After 24h, Map2 expression is already 6-fold compared to proliferating cells, whereas the expression of Tuj1 is a little delayed. Its expression starts to increase after 48h (3-fold compared to proliferating cells), and then 6-fold after 48h. This was expected as Map2 is described as an early marker of neuronal differentiation.

The presence of GSK-3β inhibition to hNPC during differentiation had no effects on the expression of neuronal markes. Interestingly, the expression of Tuj1 was significantly reduced by SB-216763 compared to DMSO-control cells. AB199 had no influence on the expression of these two markers.

The next step was to investigate the influence of the GSK-3β inhibitors on neuronal differentiation on protein level. Therefore, hNPCs were differentiated for 3d in the presence of inhibitors, then immunocytochemically stained for βIII-tubulin and analyzed by flow cytometry (2.2.3.6).

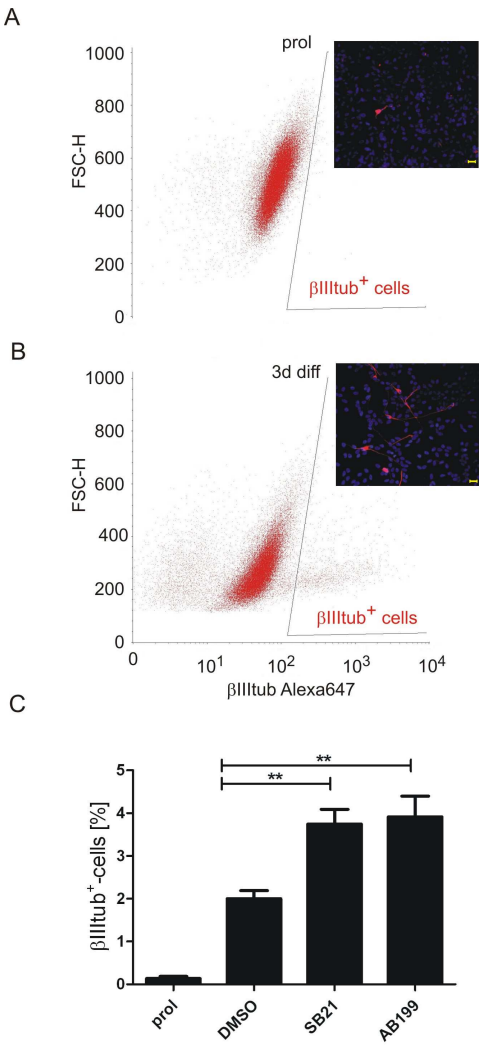

Figure 24: Influence of SB-216763 (SB-21) and AB199 on neuronal differentiation.
hNPCs were differentiated in the presence of the compounds or DMSO. Expression of βIII-tubulin was determined by immunocytochemistry (insert A and B, scale bar indicates 20 µm) and quantified by flow cytometry (A and B). After 3 days of differentiation 2.0 ± 0.2% βIIItub$^+$ cells were detected under control conditions. The treatment of cells with SB-216763 or AB199 resulted in an increase of βIIItub$^+$ cells up to 3.2 ± 0.4 % and 3.7 ± 0.7%, respectively. Data represent mean ± SEM (N = 6). *p<0.05.

Proliferating cells expressed βIII-tubulin at a very low percentage (0.02%) which is most likely caused by spontaneous differentiation (Fig. 24c). Previous experiments have shown that the expression of βIII-tubulin in hNPCs peaked after 3d (Morgan et al, 2009). In this study 2% βIIItub$^+$ cells after 3d of differentiation were detected (Fig. 24c). This number

could be increased significantly by the addition of SB-216763 to 3.2% βIIItub^{+} cells. Conditioning of the cells with AB199 could even augment the effect caused by SB-216367, whereas 3.7% of the cells were positive for βIII-tubulin. This effect was significantly different to control cells, but not to SB-216763-treated cells.

3.3.3 Different effects of GSK-3β inhibitors on apoptosis in hNPC

As demonstrated, the treatment of the hNPCs with GSK-3β inhibitors results in an increase of βIII-tubulin^{+}-cells. This raised the question whether this effect is based on an enlarged progenitor pool or if GSK-3β inhibitors act in a neuroprotective way. It has been described that GSK-3β may promote apoptosis (Bijur et al, 2001 und 2000). Inestrosa and co-workers demonstrated that in Alzheimer's disease, the inhibition of GSK-3β by lithium and PKC activators obviate neuronal damage which is caused by Amyloidβ-toxicity (De Ferrari et al., 2003; Garrido et al., 2002). Thus, we wanted to know if early differentiation of hNPCs is connected to apoptosis and how this might be influenced by GSK-3β inhibitors.

hNPCs were differentiated in the presence of GSK-3β inhibitors up to 12h. Cells were harvested every 3h and then stained with Annexin and PI (Propidiumiodide) (2.2.3.7). Annexin binds to phosphatidylserine, which is translocated during apoptosis from the inner side of the membrane to the outer side. PI can enter the cells through the disrupted membrane of dead cells and binds to DNA. One can distinguish between alive. (Annexin-/PI-), early apoptotic (Annexin+/PI-), and late apoptotic or dead cells (Annexin+/PI+).

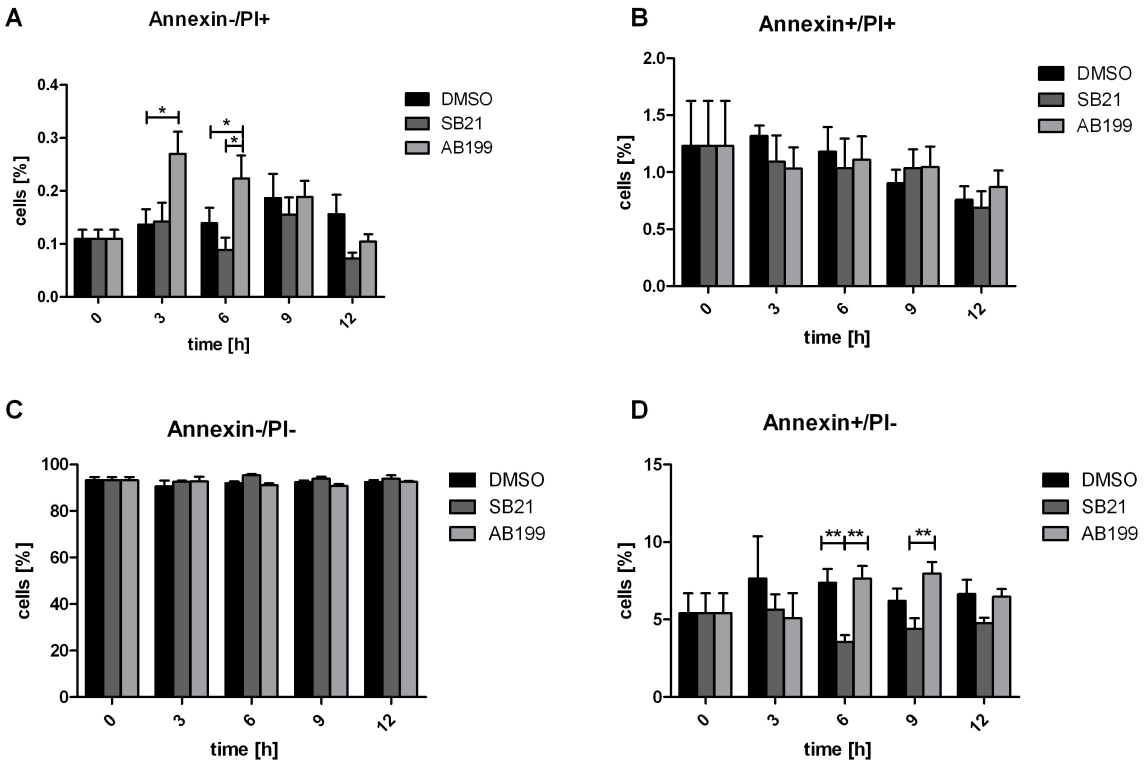

Figure 25: Apoptosis during early differentiation of hNPCs is influenced differently by GSK-3β inhibitors.
Cells were cultured under differentiation conditions up to 12h in the presence of 3µM SB-216763 (SB21), AB199 or the equivalent amount of DMSO. Then cells were harvested, stained with Annexin/PI and measured by flow cytometry. SB-216763 reduced significantly the number of early apoptotic cells. Data represent mean +/- SEM (N=3-10). Values were significantly different at *p< 0.05 or **p<0.01.

hNPCs showed no increase of early apoptotic cells after differentiation started, the number of late apoptotic cells decreased from 1.2% in proliferating cells to 0.9% after 12h of differentiation (Fig. 25b). SB-216763 significantly reduced the number of Annexin+/PI- cells after 6h from 7.4% to 3.5%, whereas AB199 did not imitate this effect (7.6% Annexin+/PI- cells) (Fig. 25d). After 9h, the number of early apoptotic cells was still significantly lower in SB-216763 cells than in AB199-treated cells. The number of late apoptotic cells was not influenced by GSK-3β inhibitors.

4 Discussion

Human neural progenitor cells offer great potential to study proliferation and differentiation. An immortalized cell line has the advantage of a “never-ending source” and of high standardization.

A plethora of studies have shown that Wnt/β-catenin signaling is involved in neural progenitor proliferation and differentiation in the murine or rat system (Castelo-Branco et al., 2003; Hirsch et al., 2007; Shimizu et al., 2008; Kunke et al., 2009). Artificial stimulation of Wnt/β-catenin signaling by GSK-3β inhibition would enable us to study the influence of this signaling pathway in human neural progenitor cells. Another use for GSK-3β inhibitors is the investigation of any disease which is linked to a malfunction of GSK-3β.

Therefore, we wanted to develop a novel screening system to find new GSK-3β inhibitors and then evaluate their effect in hNPCs.

4.1 GSK-3 inhibition and Wnt signaling in hNPC

Previous data have shown that hNPCs respond to Wnt signals, either by blocking Wnt/β-catenin signaling by treatment with DKK1 (Ahn et al., 2008) or activation by stimulation with Wnt-3a (Soen et al., 2006 Hübner et al., 2010). But the effect of the inhibition of GSK-3β on the activation of the Wnt/β-catenin signaling in hNPCs remains unclear.

ReNcell VM have been shown to be capable of activating Wnt/β-catenin signaling by overexpression of a stabilized form of β-catenin. Thus, this cell line is suitable to study the influences of GSK-3β inhibition on Wnt/β-catenin signaling on hNPC and its outcome on cell proliferation and differentiation.

4.1.1 GSK-3 inhibition and activation of Wnt cascade in hNPCs

It is widely described that NPCs respond to GSK-3β inhibition with a β-catenin accumulation as β-catenin is one of the key substrates of GSK-3β. Thus, it is an often used

as an indirect read-out for GSK-3β inhibition (Adachi et al., 2007; Maurer et al., 2007; Jung et al., 2008).

Treatment of hNPCs with known GSK-3 inhibitors Kenpaullone and SB-216763 resulted in an up-regulation of β-catenin (Fig. 5). In addition, native differentiation also increased the amount of β-catenin, but this effect was more prominent in the presence of Kenpaullone and SB-216763, indicating that the differentiation process is connected to GSK-3β inhibition. To investigate whether new compounds, structurally similar to known GSK-3β inhibitors, could increase β-catenin accumulation, nearly 200 novel small molecules were tested regarding their ability to accumulate β-catenin. Most of them had a high structural similarity to the known GSK-3β inhibitor SB-216763, whereas others included other structures that have been shown to be biologically active. Substances were provided by the Leibniz-Institute for Catalysis (LIKAT) and any analyses regarding structure and corresponding biological activity were performed by LIKAT. Several substances showed a β-catenin accumulation in the same range than known GSK-3β-inhibitors, but the first hit, namely AB199, was selected to investigate the influence of GSK-3β inhibition on Wnt/β-catenin pathway in hNPC and its influence on hNPC proliferation and differentiation.

AB199 enhanced β-catenin accumulation in the same range as Kenpaullone (Fig. 6). It was of interest whether or not structural modification of this 3-(4-Fluorophenylethylamino)-1-methyl-4-(2-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione could even improve this effect or if there is any relation between structure and biological action. The effectiveness of AB199 could be ascribed to the amine moiety, which is an additional hydrogen bonding motif. In addition, it has been reported that biologically active substances often benefit from the presence of fluorine substituents due to improved metabolic stability, bioavailability and protein–ligand interactions of the fluorinated compounds (Hagmann, 2008; Shah and Westwell, 2007). Thus, the substitution with one or more fluorine atoms (Woo et al., 2008; Memic and Spaller, 2008) and more specifically, the incorporation of the 4-fluorophenethylamine unit (Schenone et al., 2008; Saha et al., 2005) has led to an increased biological activity of small molecule therapeutics.

Derivatization of AB199 resulted in no further improvement regarding β-catenin accumulation, indicating a specific relation between structure and action.

The working concentration of an inhibitor is crucial for every experiment. Too high a concentration could cause unspecific binding and undefined side effects as well as

potential toxicity. On the other hand, a concentration which is too low could not be sufficient. SB-216763 and AB199 were tested in concentrations from 0.1-10µM regarding potential cytotoxicity in a cell metabolic activity, but none of the tested concentrations seemed to harm the cells. Previous experiments revealed a most prominent effective β-catenin accumulation for SB-216763 with 3µM (Christian Lange, unpublished data). AB199 showed an increasing β-catenin accumulation from 0.1-3µM with a maximum at 3µM but followed by a rapid decrease from 5µM to 10µM. An IC_{50} of 3.8µM was calculated but this specific kinetic offers only a very narrow therapeutic window. These effects cannot be explained with a potential cytotoxicity as this has been ruled out with experiments regarding metabolic activity (Fig. 14). Another possibility would be that β-catenin is also phosphorylated by other kinases such as CK1, PKA (protein kinase A), AKT or JNK2 (Verheyen and Gottardi, 2009) and thus sufficiently primed for degradation. This idea is rather unlikely as the same bell-shaped dose-response curve was observed in an *in vitro* kinase activity assay, where a recombinant enzyme was used. The IC_{50} for SB-216763 was 92nM, slightly higher than the given value in the literature (34nM, Coghlan et al., 2000). This difference could be explained by the fact that we used a luminometric detection system, whereas Coghlan and co-workers used radioactive detection. AB199 showed an even lower IC_{50} with 53nM. These data were be confirmed in a cell-based kinase-assay, where both substances at the same concentration inhibited GSK-3β in the same range. In both assays a bell-shape dose-respond curve was observed. This type of dose-response curve is well described for PKC (Govoni et al., 1992; Qiu et al., 1998; Moriguchi et al., 2002), but these data are generated in cell-based assays, where other cellular components can be involved in enzyme regulation, like feedback-regulation of downstream components within signaling pathways. Involvement of other kinases or feedback-regulation could explain the downregulation of β-catenin accumulation in the presence of high inhibitor concentrations we observed, but not the re-activation of GSK-3β in the *in vitro* kinase assay. As this assay is performed independent of any cellular influences, the reason for the bell-shaped dose-response relationship must be within the enzyme/activator kinetics itself. One possibility would be that AB199 agglutinates at a higher concentration and thus a binding to the ATP binding site would be impossible, therefore re-activating GSK-3β. Another explanation might be a change within the enzyme conformation after the binding of AB199. The conformation change could open

an additional ATP binding side which then re-activates GSK-3β. However, up to now, there is no information existing about GSK-3β as an allosteric enzyme.
The combination of a constant concentration of SB-216763 with variable concentrations of AB199 did not enhance β-catenin accumulation. Again, it could be observed that higher concentration of GSK-3β inhibitors seemed to abolish the inhibitory effect of lower concentrations, indicating that high concentrations of AB199 are crucial for the activity of GSK-3β.

As a consequence of the accumulation in the cytosol, β-catenin is translocated to the nucleus. The shuttling process has not been well described yet. Early suggestions that this process might be triggered by a nuclear location sequence (NLS) could not be verified as β-catenin does not possess a NLS (Fagotto et al., 1998). The translocation seems to be importin-independent but might involve direct interactions with nuclear pore proteins. Importin recognizes and binds NLS, thus enabling a protein to enter the nucleus (Henderson and Fagotto, 2002). This process could not be visualized in hNPCs, which is due to the growth pattern of ReNcell VM cells. ReNcell VM cells need to grow to 80% confluence to be differentiated and then form a dense network which makes it difficult to analyze single nuclei. Immunocytochemical stainings for β-catenin are rather diffuse (Fig. 14). To overcome this ST14A cells have been used to prove the accumulation of β-catenin. ST14A cells are derived from rat striatum and display a flat morphology with grand nuclei. In addition, they have been used before to visualize nuclear β-catenin shuttling (Lange et al., 2006). Both, SB-216763 and AB199 triggered perinuclear β-catenin accumulation, indicating that both GSK-3 inhibitors promote the Wnt/β-catenin signaling pathway in hNPC.

The intranuclear consequence of β-catenin translocation is the activation of TCF-dependent transcription. This can be measured by a reportergene-assay, where a TCF/LEF reporter luciferase system is used. Transfected, proliferating cells showed no transcriptional activity, which is not unusual for NPCs (Lie et al., 2005; Wexler et al., 2009) but could also be below the detection limit. Previous data revealed that only 1.2% of proliferating ReNcell VM display TCF-dependent transcription, using a GFP-reporter construct (Jana Frahm, unpublished data). This could be a result of the short lifetime of

luciferase of only 2h. Downregulating of Wnt/β-catenin signaling during cell cycle phases is crucial for the proper development of other types of dividing progenitors (Aulehla et al., 2003), but only a certain proportion of asynchronously proliferating cells will exhibit canonical activity which cannot be detected due the short lifetime of the luciferase. Surprisingly, differentiation as well as additional stimulation with GSK-3β inhibitors failed to induce TCF-activity (data not shown). This effect was be ruled out by co-expressing a stabilized form of β-catenin, S33Y, suggesting that a certain threshold of TCF/luciferase-activity needs to be reached. The co-transfection successfully promoted TCF-activity and additional effects of GSK-3β could be distinguished (Fig. 16). TCF-activity increased under differentiation conditions over the time after an initial decrease after 6h. Inhibition of GSK-3β enhanced TCF-activity compared to control cells, whereas the kinetics seemed to be shifted. SB-216763-dependent effects reached a maximum after 18h, while AB199 treated cells exhibited maximal TCF-activity after 24h. In general, TCF-activity seemed to be higher in AB199 treated cells than in SB-216763 treated cells. Several factors could be responsible for this. AB199 could also prime the nuclear shuttling of β-catenin or the kinetic of TCF-activity could be influenced by both substances in a different way.

The fact that only a small population of hNPCs (6% after stimulation with stabilized β-catenin) can activate TCF-mediated transcription is also described for other NPCs. Hirsch et al. (2007) report that treatment with SB-216763 or Wnt3a resulted in a TCF-activation in mNPCs of 14% and 8%, respectively. It is most likely that these effects are not cell line dependent or based on experimental problems rather than a physiological behavior, as the transcription efficiency of hNPCs is very high (higher than 90%) and any effects of secreted inhibitors have been excluded by the use of stabilized β-catenin.

The nuclear translocation and the induction of TCF-activity are followed by the transcription by Wnt specific target genes. To this purpose, different target genes were analyzed regarding their transcriptional activity as a consequence by GSK-3β inhibition, namely Axin2, LEF1, c-Myc, Kremen 1 and 2, RSPO 2 and 3, Dkk1 and CCDN1, but only the expression of Axin2, Kremen 1, c-Myc, Dkk1 and CCDN1 could be detected in hNPC. Axin2 has been described as a negative feedback regulator of Wnt signaling (Jho et al., 2002). Dkk1 inhibits Wnt/β-catenin signaling and was observed as a Wnt target gene in cancer cells (Niida et al., 2004). CCDN1 is a regulator of the cell cycle and is communicated as

Wnt target gene (Shtutman et al., 1999). C-Myc is the cellular homologue of the viral v-Myc; the expression of the proto-oncogene can be induced by Wnt signaling (He et al., 1998).

In the hNPCs used in this study the start of differentiation upregulated the expression of Axin2 and Kremen 1 (Fig. 20a and e) with maximum expression after 96h. The expression of c-Myc was reduced slightly by the induction of differentiation but then also increased. In contrast to this, c-Myc, CCDN1 and Dkk1 expression were downregulated with the start of differentiation (Fig. 20b and d). This was expected as these genes are known to promote cell proliferation and conditions are now changed to differentiation conditions lacking growth factors. These findings correspond with the fact that the expression of these genes can be induced by FGF and EGF (Kurz et al., 2003; Corbetta et al., 2007). Interestingly, Axin2 expression was increased up to 30-fold compared to proliferating cells whereas the other genes responded with expression changes with only up to 5-fold. This corresponds with the finding that Axin2 contains 8 TCF/LEF binding site consensus sequences in its enhancer region and can be activated in various tissues (Jho et al., 2002).

The main interest of these experiments was to find out whether or not the treatment of hNPC with GSK-3 inhibitors manipulates the expression of Wnt target genes. The expression of Kremen1 and Dkk1 was not influenced by the presence of small molecules. C-Myc and CCDN1 showed some slight increase in gene expression in the presence of SB-216763 but not with AB199. This effect was not constant over the time. Only Axin2 responded with a steady increase in gene expression to the presence of GSK-3β inhibitor SB-216763. But here, as well as for CCDN1 and c-Myc, this effect could not be observed for AB199. These data are on the same line as the findings of Hirsch and colleagues (2007). They analyzed murine NPC and found that only a few of described Wnt target genes respond to stimulation of the Wnt/β-catenin pathway by the inhibition of GSK-3β, namely Axin2, Nkd1 (Naked 1) and TCF1. Expressions of other Wnt target genes could be detected, but were not influenced by GSK-3β inhibition. The authors also spotted an increased expression of LEF1 by SB-216763 which we could not detect at all. In contrast to this, an enhanced expression of CCDN1 by SB-216763 was observed whereas Hirsch et al. monitored no difference. This could be due to differences within the cell line origin. Interestingly, the novel GSK-3β inhibitor did not influence the expression of Wnt target genes, whereas the upstream processes, namely GSK-3β inhibition, β-catenin

accumulation, translocation and TCF-activity, were manipulated in the same range as in the presence of SB-216763.

The expression of several components of the Wnt/β-catenin pathway was analyzed by TaqMan array. It was of interest whether or not differentiation of hNPCs influenced the expression of Wnt receptors and ligands and if this could be manipulated by GSK-3β inhibitors. A difference in expression of any upstream components of GSK-3β by its inhibition would suggest a positive or negative feedback loop.

Within the expression of Wnt-related receptors, an endogenous expression of several receptors was monitored which changed at the start of differentiation. Several publications describe that NPCs express a manifold pattern of Wnt receptors (Rawal et al., 2006; Ahn et al., 2008; Wexler et al., 2009). The expression of the co-receptor Ror2 caught our attention, as it was marginal detectable under proliferation conditions but displayed a tremendous increase of expression after 24h of differentiation. The role of Ror2 is cell-type and ligand specific as it can bind Wnts and thus increasing or inhibiting Wnt/β-catenin signaling (Mikels and Nusse 2006; Li et al., 2008). Its increased expression during differentiation can be explained by its role in modulating neurite growth, their branching pattern and synaptic formation (Paganoni and Ferreira, 2005; Paganoni et al., 2010).

In addition, a few Wnt ligands could be detected, namely Wnt 5a, 5b, 7a and 10b. Wnt 5a is described as an activator of the Wnt/Ca^{2+}-pathway (Slusarski et al., 1997), but it has also been shown that it can activate the Wnt/β-catenin dependent pathway in murine NPCs (Yu et al., 2005). Both Wnt 5a and 5b trigger proliferation and differentiation of chondrocytes, whereas only Wnt 5a has been demonstrated to support dopaminergic differentiation (Castelo-Branco et al., 2006; Anderson et al., 2008). The upregulation of these genes together with previous findings that ReNcell VM are capable to differentiate into dopaminergic neurons (Donato et al., 2007; Morgan et al., 2009, Ortinau et al., 2010) may suggest that the expression of Wnt 5a is connected to dopaminergic differentiation in hNPCs. Together with the upregulation of Ryk, another role for Wnt 5a could be axonal outgrowth during differentiation, as this has been described as a consequence of Wnt 5a binding to a Ryk receptor (Li et al., 2009).

Wnt 7a is expressed in various NPCs. Beside in hNPC ReNcell VM cells, it has been found in neonatal mouse NPCs (Hirsch et al., 2007), in cells of the human neural progenitor cell line HB1.F3 (Ahn et al., 2008), E.11.5-E13.5 mouse NPCs (Rawal et al., 2006) and in rat adult hippocampal progenitors (Wexler et al., 2009). However, its impact is discussed controversially as it is reported to either promote (Hirabayashi et al., 2004) or to obviate neuronal differentiation (Viti et al., 2003). Combined with Fzd7, it might be responsible for the predominantly glial-differentiation of ReNcell VM cells, as it has been shown to be expressed in glial population (Rawal et al., 2006).

Beside native differentiation, gene expression of Wnt receptors and ligands was analyzed in the presence of GSK-3β inhibitors. Neither the expression of Wnt receptors nor the expression of Wnt ligands were significantly influenced by SB-216763 or AB199. These findings are in contrast to the data of Maurer and co-workers (2007) as they report that the treatment of rat hippocampal NPCs with SB-216763 resulted in an upregulation of Wnt 5a expression and a downregulation of Wnt 7a expression. In ReNcell VM cells a decrease of Wnt 5a expression after 72h by SB-216763 was detected, but in general SB-216763 and AB199 seemed to have no effect on the expression of Wnt ligands or receptors. The difference to the data from Maurer could be explained with the different origin of the cells. The hypothesis that GSK-3β inhibition might manipulate the expression of Wnt ligands or receptors and therefore indicate a positive or negative feedback loop could not be verified in hNPCs.

In conclusion, these data suggest that Wnt/β-catenin plays a pivotal role during the differentiation process of hNPCs which can be influenced GSK-3β inhibition. The next step was to investigate the impact of GSK-3β inhibitors on hNPCs proliferation and differentiation.

4.1.2 Influence of GSK-3 inhibition on proliferation and differentiation of hNPCs

The outcome of GSK-3β-inhibition of NPCs fate seems to depend mainly on cell origin and stage. In general, murine NPCs derived from early embryonic stages respond with enhanced proliferation to GSK-3β inhibition whereas NPCs obtained from later embryonic stages show an increased differentiation.

The hNPCs used in this study displayed a doubling time of 19.8h, which is fast compared to other NPCs, mainly of murine origin, which have doubling time of 4-7 days (Doetsch et al., 1999; Imura et al., 2003; Hirsch et al, 2007). Treatment of hNPCs resulted in a deceleration of cell proliferation and an increase of cell doubling time, 24.4h for SB-26763 and 25.7h for AB199, respectively. These effects are controversially described in the literature. Hirsch and co-workers observed no effect of GSK-3β inhibition by SB-216763 on proliferation of murine NPCs derived from the neonatal cortex. In contrast, murine NPCs isolated from the subventricular zone showed an enhanced proliferation as a consequence of GSK-3β inhibition by R3303544, which is structurally very similar to SB-216763. The same effect of SB-216763 could be observed in murine NPCs derived from the telencephalon (Shimizu et al., 2008). GSK-3β inhibition has been also investigated in tumor cell lines. The size of colon tumors formed out of SW-480 cells could be reduced by treatment with SB-216763 or AR-A014418, another GSK-3β inhibitor (Shakoori et al., 2007). Mazor and colleagues demonstrated that cell proliferation in prostate cancer cells is also decreased in the presence of SB-216763 (2004). They explain these results with their findings that GSK-3β is required for androgyn receptor (AR) transcriptional activity, which is a mediator for prostate cell growth. Therefore it is most likely that this explanation does not apply to hNPCs. It is more likely that resemblance of effects of GSK-3 inhibition in tumor cells and the used hNPC cell line ReNcell VM could be a result of the immortalization of ReNcell VM cells with the proto-oncogene v-Myc, as this protein is also described as a target substrate of GSK-3β (Pulverer et al., 1994; Sears et al., 2000). C-Myc is phosphorylated by GSK-3β and therefore primed for degradation, by this process, cell proliferation can be inhibited. The cell cycle regulator Cyclin D1 has also been reported as target substrate of GSK-3β and is phosphorylated by the kinase as well and by this process prepared for degradation (Diehl et al., 1998). But as inhibition of GSK-3 would lead to a stabilization of these cell proliferation regulators, it is also improbable that these incidents are responsible for the reduced cell proliferation of hNPCs in the presence of GSK-3β inhibitors. Therefore the question arose whether the monitored effects depended on GSK-3β itself or if they were based on the activation of Wnt/β-catenin signaling. If substances that promote β-catenin accumulation diminish cell proliferation, then substances that do not influence or even suppress β-catenin accumulation should acclerate cell proliferation. Beside SB-216763 and AB199, none of the substance that

enhanced β-catenin accumulation decelerated cell proliferation and the other way around. Quite the contrary, one small molecule that depleted β-catenin accumulation also significantly downregulated cell proliferation of hNPCs (Fig. 22c). Overexpression of stabilized β-catenin could not imitate this effect (Rayk Hübner, unpublished data), indicating that a downregulation of hNPCs proliferation is independent of β-catenin accumulation. ReNcell VM cells are characterized by a very low doubling time, thus it could be impossible to even further accelerate cell proliferation. Additional experiments independent of this work together with the here presented data support the idea the GSK-3β-mediated inhibition of cell proliferation is independent of Wnt/β-catenin signaling, but is probably caused by an interaction of GSK-3β and Notch signaling. This interaction is part of a different PhD thesis and was therefore not further investigated within this work.

Some studies propose that there are different pools of GSK-3β, an Axin-bound GSK-3β that is responsible for β-catenin degradation and another pool of free GSK-3β which interacts with other pathways, such as PI3K or Notch (Ng et al., 2009; Wu and Pan, 2009). Inoki and co-workers (2006) present that GSK-3β regulates the TSC2- (tuberous sclerosis complex2) mTOR (mammalian target of rapamycin) pathway in the presence of Wnt, but apart from β-catenin. As this pathway is associated with the regulation of cell growth, it might explain why GSK-3β inhibition reduces hNPCs proliferation in a way independent from of β-catenin.

Treatment of hNPCs with GSK-3β inhibitors resulted in a significant increase of βIII-tubulin positive cells. This has been observed with other GSK-3β inhibitors in other NPCs from later developmental stages, too. Castelo-Branco and co-workers (2004) treated progenitors from the rat ventral midbrain with KP and I3M and reported an increase in neurons as well as dopaminergic neurons. Maurer and colleagues (2007) demonstrated that GSK-3β inhibition via SB-216763 in rat hippocampal NPCs results in enhanced neuronal differentiation. They concluded that this increase is a result from the enhanced transcriptional activation of β-catenin dependent target genes. This theory does not apply to hNPCs, as the upregulation of Wnt-5a and downregulation of Wnt-7a expression by GSK-3β inhibition was not confirmed in hNPCs.

In contrast, NPCs from early developmental stages show an increased proliferation and a decrease in neuronal differentiation when treated with GSK-3β inhibitors (Adachi et al., 2007; Shimizu et al., 2008). Several other studies describe that activation of Wnt/β-catenin pathway by Wnt3a promotes neuronal differentiation of murine NPCs from later stages but not from early, proliferating stages (Hirabayashi et al., 2004; Israsena et al., 2004; Muroyama et al., 2004; Viti et al., 2003).

But is this increase of βIII-tubulin positive cells based on a direct influence of GSK-3β inhibition-mediated activation of Wnt/β-catenin signaling, an enhancement of proliferation and thus enlarging of the neural progenitor pool or a neuroprotective effect of GSK-3β inhibition? The proneural genes Neurogenin 1 and 2 (Ngn1 and Ngn2) show an enhanced expression in the presence of stabilized β-catenin (Hirabayashi et al., 2004; Israsena et al., 2005). This would indicate a direct influence of Wnt/β-catenin on neurogenesis in mNPCs. In contrast, Hirsch et al. (2007) could not demonstrate a connection between cells that express the neuronal marker βIII-tubulin and cells that show TCF-activity. The increase of proliferation as a consequence of the activation of Wnt/β-catenin signaling, i.e. an extension of the neural progenitor pool would be another possibility, as Wnt signaling promotes proliferation of neural precursors from the rat hippocampus (Li et al., 2005). But this does not seem to apply to hNPCs of theReNcell VM cell line, as activation of Wnt/β-catenin signaling by Wnt3a resulted in a slight decrease in proliferation whereas overexpression of a stabilized form of β-catenin had no effect (Rayk Hübner, unpublished data). In addition, GSK-3β inhibition actually downregulated cell proliferation.

Neurogenesis is tightly connected to apoptosis, as nearly half of the neuronal progenitor pool undergoes apoptosis caused by neuronal network formation processes and synaptic selection processes (Burek and Oppenheim, 1999). The Wnt/β-catenin pathway plays a crucial role for apoptotic processes during neuronal development. Wnt-mediated inhibition of c-myc-triggered cytochrome c-release is described to be anti-apoptotic (You et al., 2002; Kanei-Ishii et al., 2004). Other data suggest that β-catenin stabilization causes massive apoptotic cell death in the murine neural crest (Hasegawa et al., 2002). GSK-3β is linked to either pro- or anti-apopotic processes. On the one hand, it can directly phosphorylate Bax, a co-factor of the tumorsupressor protein p53, thus activating the

mitochondrial, intrinsic apoptotic signaling pathway. On the other hand, GSK-3β can promote the extrinsic apoptotic-signaling pathway by inhibiting caspase-8 (Beurel and Jope, 2006).

Induction of differentiation resulted in a slight increase of early-apoptotic cells. This effect was constant over the first 12h of apoptosis. Interestingly, SB-216763 reduced the number of Annexin+/PI- negative cells during early differentiation, whereas AB199 could not achieve the same effect. SB-216763 has been shown to protect cerebellar granule neurons and sensory ganglionic neurons from cell death induced by either trophic factor withdrawal or PI 3-kinase inhibition (Cross et al., 2001). PI3K is a key enzyme of a cell survival pathway and upstream-regulator of GSK-3β (Facci et al., 2003). In addition, it has been shown that GSK-3β inhibition protects neurons from cell death by the stabilization of two GSK-3 targets, β-catenin and Tau (Cross et al., 2001), indicating that GSK-3β inhibition is a crucial tool for neuronal apoptosis. GSK-3β inhibition by SB-216763 has been demonstrated to block Bax and Caspase-3 activation as a consequence of the removal of growthfactors in murine NPCs (Eom et al., 2007). Maurer and co-workers (2007) identified GSK-3β as a key-regulator regarding differentiation and survival of rat NPCs. Treatment of rat NPCs with GSK-3β inhibitor SB-216763 resulted in an upregulation of neuronal differentiation as well as reduced apoptosis, evidencing that the increase of neurons is based on enhanced cell survival during differentiation. As in this study a downregulation of early apoptotic processes by inhibiting GSK-3β with SB-216763 during differentiation of hNPCs was observed, these data suggest, too, that GSK-3β inhibition results in an increasing cell survival during differentiation, thus expanding the number of neurons. Surprisingly, AB199 did not counterfeit this effect, indicating that there might be a different, not neuro-protective effect for the increase in βIII-tubulin cells.

4.2 Development of a multi-screening system for novel GSK-3 inhibitors

Multi-level screenings have gained more and more interest within the last years, whereas the targets vary. They are either focussed on specific targets, like pathway inhibitors/activators, or on the cell fate, disregarding the cell processes they manipulate. Most screenings include a broad, rather unspecific screening, followed by several, more

and more specific, assays. Borowiak et al. (2009) screened for small molecules that promote endodermal differentiation by using a fluorescent Sox17-dsRed-reporter. Sox17 is a marker and effector specific for endodermal differentiation. A secondary screen evaluated the function of potential hit substances to promote formation of homogeneous clusters of Sox-17-dsRed+ epithelial cells in different mouse cell lines. As a last step, they identified two hit substances as modulators of Smad2-phosphorylation, indicating the substances induce Nodal synthesis and secretion. Nodal is a member of the TGF-family and involved in cell differentiation. Another screening for substances inducing Sox17-expression revealed a substance, namely stauprimide, that primes ESC for ectoderm, endoderm and mesoderm differentiation by interacting with NME-3, a cMyc interacting transcription factor (Zhu et al., 2009). The emergence of iPSC in 2006 (Takahashi and Yamanaka) opened another field for novel small molecules that could either substitute one or more of the oncogenic transcription factors or promote the low transcription efficiencies. The known GSK-3β inhibitor Kenpaullone has been identified out of 50,000 substances to substitute the transcription factor Klf4 (Lyssiotis et al., 2009). They used a Nanog-Luciferase reporter strain as a read-out for iPSC formation, as Nanog is an important factor for maintaining the undifferentiated state and is inactivated in somatic lineages. Interestingly the replacing effect of Kenpaullone is independent of its ability to inhibit GSK-3β, as other, more specific inhibitors, could not mimic this effect.

Most screenings systems used to find Wnt modulating agents search for Wnt/β-catenin signaling inhibitors, as Wnt signaling is often constitutively active in several tumors (preferential in the intestine), which are mostly due to the loss of function of APC, Axin1/2 or stabilizing mutations of β-catenin (Clevers, 2006). GSK-3β is an interesting pharmalogical target, as it is not only involved in cancer but also in Alzheimer's disease (Phiel et al, 2003), bipolar disorders (Gould, 2006) or diabetes (Cohen and Goedert, 2004). Different studies have shown that the activation of Wnt/β-catenin in NPCs from later developmental stages promotes neuronal differentiation (Hirabayashi et al., 2004; Muroyama et al., 2004; Hirsch et al., 2007; Hübner et al., 2010). This could be also demonstrated when NPCs were treated with GSK-3β inhibitors (Castelo-Branco et al., 2004; Maurer et al., 2007). Hence, one aim of this study was to develop a multi-level screening system to identify novel GSK-3β inhibitors that elevate neuronal differentiation in hNPCs. The initial screening should cover a broad range, thus an ELISA for β-catenin

was chosen. β-catenin is suitable as a read-out parameter for GSK-3β inhibition, as its accumulation is a consequence of GSK-3 inhibition. An ELISA can be performed in 96-well format and is easy to standardize. The next step should cover the activation of the Wnt/β-catenin pathway as well as the proof that the β-catenin accumulation is due to direct GSK-3β inhibition. An in-vitro GSK-3 binding assay with a recombinant enzyme is a clear evidence for a GSK-3β inhibitor, as any cellular components that could cause side effects are excluded. This system could be performed with luminometric detection (Baki et al., 2007), which is of great advantage in comparison to any radioactive detection system. The calculated IC_{50} for SB-216763 as GSK-3β inhibitor of 92nM is in the same range of the given literature value of 34nM (Coghlan et al., 2000). The differences could be caused by the different detection systems, as Baki et al. (2007) also reported slight differences between the radioactive literature IC_{50}, radioactive in-house measurements and luminometrically performed assays. As this assay can be also performed in 96-well or smaller format, which would be an interesting approach for a high-throughput screening. A downstream consequence of GSK-3β inhibition and cytosolic β-catenin accumulation is the nuclear translocation. The nuclear β-catenin accumulation is very hard to visualize and detect in the used cell line, which is caused by the dense growth pattern of ReNcell VM cells. In contrast the TCF-dependent transcriptional activity as well is described as a read-out for GSK-3β inhibition and therefore the activation of Wnt/β-catenin signaling (Liu et al., 2005; Zhang et al., 2007; Chuang et al., 2010; Ewan et al., 2010). Here, an increase of TCF-activity in the presence of GSK-3β inhibitors compared to control cells was shown, though the co-transfection of a stabilizing form of β-catenin is necessary. Although ReNcell VM cells can be transfected at a very high efficiency (higher than 90%), a stable transfection would improve this assay as transfection of this cell line is connected to a high variance. The expression of Wnt/β-catenin related target genes revealed not to be suitable as a read-out for GSK-3β inhibitors as we could not detect any differences in gene expression mediated by GSK-3β inhibition. Only SB-216763 could influence Axin2 expression but not AB199. This is on the same line as the data of Hirsch and-co-workers (2007) as they report that only a few genes respond to GSK-3β inhibition. Not only the expression of Wnt target genes but also the expression of Wnt ligands or Wnt receptor was not affected by GSK-3β inhibition. The best targets for evaluating the effects of β-catenin accumulation modulating substances in hNPCs would thus be firstly, an *in vitro*

kinase assay to test whether the increase in β-catenin accumulation is therefore based on GSK-3β inhibition or not and secondly, to measure the TCF-mediated transcription activity, as this is a well-described assay to examine the activity of Wnt/β-catenin signaling.

Of final interest would be if the new identified GSK-3β inhibitors can improve neuronal differentiation in hNPCs. The detection of neuronal markers via flow cytometry enables a much higher throughput than immunocytochemical staining combined with manual counting. The detected cells positive for neuronal markers are comparable in both systems (data not shown). The influence of GSK-3β inhibitors on cell proliferation or apoptosis are of interest regarding the mechanism of action but not relevant for a screening system.

In conclusion, the following screening system for the development and evaluation novel GSK-3β inhibitors is proposed:

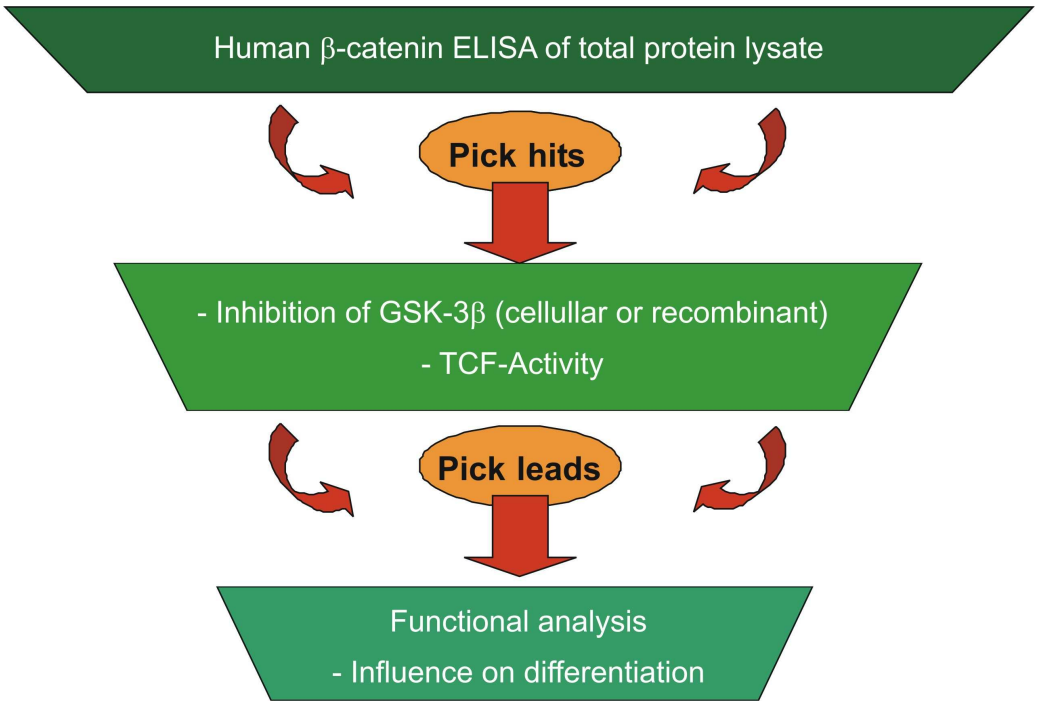

Figure 26: Final multi-screening approach for novel GSK-3β-inhibitors.

The primary screen evaluates the accumulation of β-catenin in the cell as a consequence of potential GSK-3β inhibition. The secondary screening step investigates on the one hand the direct inhibition of GSK-3β by novel small molecules; on the other hand, the TCF-

dependent transcription activity is analyzed as a downstream target of GSK-3β inhibition and Wnt/β-catenin signaling. The third screening step covers the impact of GSK-3β inhibition on neuronal differentiation of hNPCs.

5 Summary

Wnt signaling is crucial for the developing CNS, wherein GSK-3β is the key enzyme. It has been shown that Wnt/β-catenin signaling can promote neurogenesis in NPCs from later developmental stages. As the activation of Wnt/β-catenin cannot only be induced by binding of a Wnt ligand, but also by the inhibition of GSK-3β, small molecule inhibitors for GSK-3β are of great interest. In addition to this, GSK-3β is an interesting pharmacological target as it is involved in various diseases, such as diabetes, cancer, bipolar disorders or Alzheimer's disease.

The human neural progenitor cell line ReNcell VM can differentiate into astrocytes, oligodendrocytes and functional neurons and has been also shown to be appropriate for Wnt signaling studies.

The aim of this study was to develop a multi-level-screening system for novel GSK-3β inhibitors and to analyze their impact on proliferation and differentiation on hNPCs.

This study has demonstrated for the first time that β-catenin is present in hNPCs and can be accumulated by the treatment of hNPCs with GSK-3β inhibitors. Accumulation of β-catenin is subsequently followed by nuclear translocation and activation of TCF-dependent activity. The expression of different Wnt target genes as well as gene expression of Wnt receptors and ligands could be proven. Conditioning of hNPC with GSK-3β inhibitors did not implicate changes in the expression profile of Wnt components, thus indicating that the inhibition of GSK-3β causes no feedback loop by activating any upstream components of the Wnt/β-catenin pathway. The known GSK-3β inhibitor SB-216763 as well as the newly discovered GSK-3β inhibitor AB199 triggered these processes with the same efficiency, whereas AB199 displayed a lower IC_{50} value for GSK-3β inhibition than SB-216763, indicating a higher specificity.

Cellular consequences of GSK-3β inhibition were a reduced cell proliferation, whereas an enhanced neuronal differentiation was observed. In the case of SB-216763, this effect was most likely based on a neuro-protective effect, as SB-216763 reduced the number of early apoptotic cells during early differentiation. As AB199 did not show the same anti-apoptotic effects, its mechanism of action remains unclear, a possible explanation would be an increase in the pool of NPCs.

The results obtained propose the following read-outs for a screening approach for novel GSK-3β inhibitors:

1. Accumulation of β-catenin
2. a) Inhibition of GSK-3β
   b) Activation of TCF-dependent activity
3. Influence on neuronal differentiation

This study also revealed some cytotoxic small molecules. Their impact on the proliferation and viability of tumor cells and the mechanisms of action are currently investigated in an independent PhD thesis.

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7 Appendix

Acknowledgements

Many people contributed to this work in various ways and I would like to thank them all:

First of all Prof. Arndt Rolfs, for giving me the chance to work in his lab, his support and interest in my work

Dr. Stefanie Ortinau and Dr. Moritz Frech shared my supervision during the 3 years of my PhD. Steffi, you were a great teacher in “How to write a paper”. Moritz, thanks for discussing my data, helping with figures “Corel ärgert mich schon wieder” and both for your overall support.

The past and present AKos lab members: Andrea, Anne2, Annett, Caro, Christian, Ellen, Jan, Jana, Jiankai, Lea, Michi, Norman, Orianne, Peter, Ramona, Rayk, Steffen, Venkata and Xin
Andrea is the Queen of flow cytometry, establishing so many different markers from what we all benefit. And for sharing sherbets;)
Anne2 motivated me for our Friday-library-thesis-writing-session
Christian “my” PhD student. Thank you for your great enthusiasm in this project
Jana introduced me into the world of Wnts and the life as PhD student
Jan for discussing data or experiments from the sight of a “non-Wnt fellow”. And of course for so many papers
Peter for the proof-reading of my manuscripts
Rayk has a question to every answer “ Ich hab da mal gelesen, dass…” or “Hast du mal folgendes probiert?”
Ramona and Lea, our student assistants. Especially Lea, who took the load of me during the final stage of me thesis
And finally Norman and Ellen, who are awesome technicians and the perfect lab managers. And beside this, great friends. Thank you!

The Leibniz-Institute for Catalysis, Prof. Matthias Beller and his group, for providing the small molecules. Especially Anahit Pews-Davtyan and Anne Brennführer for explaining the chemistry behind and writing papers.

The research training group “dIEMoSIRIS” for giving the chance to get as an associated PhD student an insight in a, for me, total new field of science.

Christiane, as my friend and PhD fellow sufferer. Even if we don’t meet that often or only in trains;)

Tante Lore, for your brave proof-reading of my thesis. “Oh, ich verstehe, da fehlt kein Buchstabe, sondern Wnt scheint ein Eigenname zu sein”.

Daniel, for your overall support and encouragement, 1200 km of train journeys on one weekend, bearing my steady frustration, weekends in the lab, Spitzen stecken. Thanks for everything!

And finally my family, my parents Ursula und Helmut Schmöle and my sister Isabelle, for your support and encouragement during all periods of my life. Nonna, who would be very proud of me but left us shortly before the end.

Thank you all!

Publications

Published or Accepted Journal Publications

Pews-Davtyan A., Tillack A., **Schmöle A-C**., Ortinau S., Frech MJ., Rolfs A., and Beller M. "A new facile synthesis of 3-amidoindole derivatives and their evaluation as potential GSK-3β inhibitors", Org. Biomol. Chem, 2010, 8, 11-49-53

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Submitted Publications

Schmöle A-C., Rolfs A., and Frech MJ "Usage of Small Molecules in Stem Cell Research" submitted as invited review at Curr Pharm Biotech, 2010

Abstract and Posters at Scientific Meetings

Schmöle A-C., Pews-Dayvtan A., Brennführer A., Tillack A., Frahm J., Beller M., Rolfs A., und Ortinau S. "Glycogen Synthase Kinase-3β via the Accumulation of β-catenin in Human Neural Progenitor Cells", 5th Stem Cell School for Regenerative Medicine, Berlin, Germany, 2008

Schmöle A-C., Brennführer A., Pews-Dayvtan A., Tillack A., Frech MJ., Beller M., Rolfs A., und Ortinau S. "Inhibition of Glycogen Synthase Kinase-3β by novel small molecules activates Wnt signaling in Human Neural Progenitor Cells", 6th Stem Cell School for Regenerative Medicine, Odense, Denmark, 2009

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